



PATENT APPLICATION
IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Docket No: 27866/32960

PATENT APPLICATION TRANSMITTAL

Box Patent Application
Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

Transmitted herewith for filing is the patent application of

Inventor(s): 1-00 Patrick W. Gray

Title: Chitinase Materials and Methods

1. Type of Application

This new application is for a

- ☒ utility patent.
☐ design patent.

2. Application Papers Enclosed

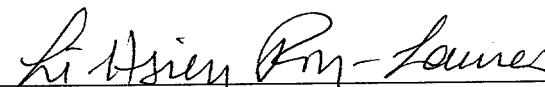
- 1 Title Page
- 47 Pages of Specification (excluding Claims, Abstract & Drawings)
- 3 Page(s) of Claims
- 1 Page(s) of Abstract
- 0 Sheet(s) of Drawings (Figs. ___ to ___)

Formal

Informal

CERTIFICATION UNDER 37 CFR 1.10

I hereby certify that this Patent Application Transmittal and the documents referred to as enclosed therewith are being deposited with the United States Postal Service on **June 14, 1996**, in an envelope addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231 utilizing the "Express Mail Post Office to Addressee" service of the United States Postal Service under Mailing Label No. EM118658816US.


 Li-Hsien Rin-Laures

08663618-082896

3. Declaration or Oath

- ☐ Enclosed
- ☐ Executed by (check all applicable boxes)
- ☐ Inventor(s)
- ☐ Legal representative of inventor(s)
(37 CFR 1.42 or 1.43)
- ☐ Joint inventor or person showing a proprietary interest on behalf of inventor who refused to sign or cannot be reached
- ☐ The petition required by 37 CFR 1.47 and the statement required by 37 CFR 1.47 are enclosed. See Item 5D below for fee.
- ☒ Not enclosed - the undersigned attorney or agent is authorized to file this application on behalf of the applicant(s). An executed declaration will follow.

4. Additional Papers Enclosed

- ☐ Preliminary Amendment
- ☐ Information Disclosure Statement
- ☐ Declaration of Biological Deposit
- ☒ Computer readable copy of sequence listing containing nucleotide and/or amino acid sequence and statement under 37 C.F.R. §1.821
- ☐ Verified statement(s) claiming small entity status under 37 CFR 1.9 and 1.27
- ☐ Associate Power of Attorney
- ☐ Verified translation of a non-English patent application
- ☐ An assignment of the invention
- ☐ Certified copy(ies) of application(s):

COUNTRY	APPLICATION NO.	FILED

from which priority under 35 USC 119 is claimed ☐ is(are) attached.

☐ will follow.

☐ Other

5. Filing Fee Calculation (37 CFR 1.16)

A. ☒ Utility Application

CLAIMS AS FILED - INCLUDING PRELIMINARY AMENDMENT (IF ANY)						
			SMALL ENTITY		OTHER THAN A SMALL ENTITY	
	NO. FILED	NO. EXTRA	RATE	FEE	RATE	FEE
BASIC FEE				\$375.00		\$750.00
TOTAL	31 -20	= 11	X 11 =	\$	X 22 =	\$242.00
INDEP.	14 - 3	= 11	X 39 =	\$	X 78 =	\$858.00
<input checked="" type="checkbox"/> First Presentation of Multiple Dependent Claim			+ 125 =	\$	+ 250 =	\$250.00
Filing Fee:				\$	OR	\$2100.00

B. ☐ Design Application (\$155.00/\$310.00)

Filing Fee: \$ _____

C. ☐ Plant Application (\$255.00/\$510.00)

Filing Fee: \$ _____

D. Other Fees

☐ Recording Assignment [Fee -- \$40.00 per assignment] \$ _____

☐ Petition fee for filing by other than all the inventors
or person on behalf of the inventor where inventor refused
to sign or cannot be reached [Fee -- \$130.00] \$ _____

☐ Other \$ _____

Total Fees Enclosed \$2100.00

6. Method of Payment of Fees

☒ Enclosed check in the amount of: \$2100.00

☐ Charge Deposit Account No. 13-2855 in the amount of: \$ _____
A copy of this Transmittal is enclosed.

☐ Not enclosed

7. **Deposit Account and Refund Authorization**

The Commissioner is hereby authorized to charge any deficiency in the amount enclosed or any additional fees which may be required during the pendency of this application under 37 CFR 1.16 or 37 CFR 1.17 or under other applicable rules (except payment of issue fees), to Deposit Account No. 13-2855. A copy of this Transmittal is enclosed.

Please refund any overpayment to Marshall, O'Toole, Gerstein, Murray & Borun at the address below.

Please direct all future communications to Li-Hsien Rin-Laures, at the address below.

Respectfully submitted,

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By:

Li Hsien Rin-Laures
Li-Hsien Rin-Laures
Reg. No: 33,547

June 14, 1996



PATENT
DOCKET NO. 27866/32960

IN THE UNITED STATES PATENT
AND TRADEMARK OFFICE

Application of:) I hereby certify that this paper is being
Patrick W. Gray) deposited with the United States Postal
Serial No: 08/663,618) Service as first class mail, postage prepaid,
Filed: June 14, 1996) in an envelope addressed to:
For: Chitinase Materials and Methods) Assistant Commissioner for Patents
Washington, D.C. 20231, on this date
August 26, 1996
Li Hsien Rin-Laures
Li-Hsien Rin-Laures

STATEMENT UNDER 37 C.F.R. §1.825(d)

Assistant Commissioner for Patents
Washington, DC 20231

Sir:

I hereby declare under 36 C.F.R. §1.825(d) that the contents of the substitute computer readable form of the Sequence Listing submitted herewith are the same as the contents of the paper copy of the Sequence Listing as originally filed pursuant to 37 C.F.R. §1.821(c).

Respectfully submitted,

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By:

Li Hsien Rin-Laures
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Reg. No: 33,547


August 26, 1996

08563618-082896



PATENT APPLICATION
ATTORNEY DOCKET NO. 27866/32960

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of:) "EXPRESS MAIL" mailing label No.
) EM118658816US
Patrick W. Gray)
) Date of Deposit: June 14, 1996
Serial No:)
) I hereby certify that this paper (or
Filed: HEREWITH) fee) is being deposited with the
) United States Postal Service
For: CHITINASE MATERIALS) "EXPRESS MAIL POST OFFICE
AND METHODS) TO ADDRESSEE" service under 37
) C.F.R. §1.10 on the date indicated
) above and is addressed to the
) Assistant Commissioner for Patents
) Washington, DC 20231
)
)
) 
) Li-Hsien Rin-Laures, M.D.
) (Reg. No. 33,547)
) Attorney for Applicant

STATEMENT UNDER 37 C.F.R. §1.821(f)


Assistant Commissioner for Patents
Washington, DC 20231

Sirs:

I hereby state that the content of the paper and computer readable
copies of the Sequence Listing, submitted in accordance with 37 C.F.R. §1.821(c)
and (e), respectively, are the same.

Respectfully submitted,

MARSHALL, O'TOOLE, GERSTEIN,
MURRAY & BORUN

BY: 
Li-Hsien Rin-Laures
Registration No. 33,547

Chicago, Illinois
June 14, 1996

08663618-082896

SOLE INVENTOR



"EXPRESS MAIL" mailing label No.

EM118658816US.

Date of Deposit: June 14, 1996

I hereby certify that this paper (or fee) is being deposited with the United States Postal Service "EXPRESS MAIL POST OFFICE TO ADDRESSEE" service under 37 CFR §1.10 on the date indicated above and is addressed to:

Assistant Commissioner for Patents, Washington, D.C. 20231

Li-Hsien Rin-Laures
Li-Hsien Rin-Laures

APPLICATION FOR
UNITED STATES LETTERS PATENT

S P E C I F I C A T I O N

TO ALL WHOM IT MAY CONCERN:

Be it known that I, Patrick W. Gray a citizen of United States, residing at 2244 38th Place East, Seattle 98112, in the County of King and State of Washington, have invented a new and useful CHITINASE MATERIALS AND METHODS, of which the following is a specification.

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CHITINASE MATERIALS AND METHODS

FIELD OF THE INVENTION

The present invention relates generally to human chitinase enzyme and more specifically to novel purified and isolated polynucleotides encoding human chitinase, to the chitinase products encoded by the polynucleotides, to materials and methods for the recombinant production of chitinase products and to antibody substances specific for the chitinase.

BACKGROUND

Chitin is a linear homopolymer of β -(1,4)-linked N-acetylglucosamine residues. This polysaccharide is second only to cellulose as the most abundant organic substance. The exoskeleton of arthropods is composed of chitin. In addition, fungi and other parasites contain chitin in their outer cell wall, where it serves important structural and protective roles. Disruption of the fungal cell wall and membrane has been a useful therapeutic strategy against fungi and parasites. For example, Amphotericin B and fluconazole exert their anti-fungal activity by affecting membrane steroids. Despite the existence of anti-fungal therapeutics, fungal infections of humans have increasingly become responsible for life-threatening disorders. See, Georgopapadakou *et al.*, *Trends Microbiol.*, 3: 98-104 (1995). The fungal species and parasites responsible for these diseases are mainly *Candida*, *Aspergillus*, *Cryptococcus*, *Histoplasma*, *Coccidioides* and *Pneumocystis*. These pathogens are particularly dangerous in immunocompromised individuals, such as patients with AIDS, patients undergoing chemotherapy, and immunosuppressed organ transplant patients.

Chitin can be degraded by the enzyme chitinase. Chitinase enzymes are found in plants, microorganisms, and animals. Bacterial chitinase helps to provide a carbon source for bacterial growth. Insects produce chitinase to digest their cuticle at each molt. In plants, chitinase is thought to provide a protective role against parasitic fungi. Chitinases have been cloned from numerous bacterial [*e.g.*, *Serratia marcescens*, Jones *et al.*, *EMBO J.*, 5:467-473 (1986)], plant [*e.g.*, tobacco,

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Heitz et al., *Mol. Gen. Genet.*, 245:246-254 (1994)], and insect [*e.g.*, wasp, Krishnan et al., *J. Biol. Chem.*, 269:20971-20976 (1994)] species.

Several proteins with low homology to bacterial, insect, and plant chitinases (less than 40% amino acid identity) have been identified in mammals, such as human cartilage gp-39 (C-gp39) [Hakala et al., *J. Biol. Chem.*, 268:25803-25810 (1993)], human glycoprotein YKL-40 [Johansen et al., *Eur. J. Cancer*, 31A:1437-1442 (1995)], oviduct-specific, estrogen-induced protein from sheep [DeSouza et al., *Endocrinology*, 136:2485-2496 (1995)], cows and humans; and a secretory protein from activated mouse macrophages [Chang et al., Genbank M94584]. However, chitin-degrading activity has not been reported for these proteins. The function of these proteins is not known, but they have been postulated to be involved in tissue remodeling. Hakala et al., *supra*, report that C-gp39 is detectable in synovial and cartilage specimens from rheumatoid arthritis patients, but not from normal humans. Recklies et al., *Arthritis Rheumatism*, 36(9 SUPPL.):S190 (1993) report localization of the C-gp39 protein to a distinct population of cells in the superficial layers of cartilage. Johansen et al., *supra*, report that measurements of YKL-40 serum levels are of value as a potential prognostic marker for the extent of metastatic disease and survival of patients with recurrent breast cancer.

Escott et al., *Infect. Immun.*, 63:4770-4773 (1995) demonstrated chitinase enzymatic activity in human leukocytes and in human serum. Overdijk et al., *Glycobiology*, 4:797-803 (1994) described isolation of a chitinase (4-methylumbelliferyl-tetra-N-acetylchitotetraoside hydrolase) from human serum and rat liver. Renkema et al., *J. Biol. Chem.*, 270:2198-2202 (February 1995) prepared a human chitotriosidase from the spleen of a Gaucher disease patient. Their preparation exhibited chitinase activity and the article reports a small amount of amino acid sequence of the protein component of the preparation (22 amino terminal residues and 21 residues of a tryptic fragment). The function of human chitinase is also unknown, but a relationship with the pathophysiology of Gaucher disease is proposed in the article. A later publication by the same group [Boot et al., *J. Biol. Chem.*, 270(44):26252-26256 (November 1995)] describes the cloning of a human macrophage cDNA encoding a product that exhibits chitinase activity. The partial

amino acid sequence reported by the group in their February 1995 article matches portions of the deduced amino acid sequence of the human macrophage cDNA product.

In view of the increasing incidence of life-threatening fungal infection in immunocompromised individuals, there exists a need in the art to identify and isolate polynucleotide sequences encoding human chitinases, to develop materials and methods useful for the recombinant production of the enzyme, and to generate reagents for the detection of the chitinase in plasma.

SUMMARY OF THE INVENTION

The present invention provides novel purified and isolated polynucleotides (*i.e.*, DNA and RNA, both sense and antisense strands) encoding human chitinase or fragments and analogs thereof; methods for the recombinant production of chitinase polypeptides, fragments and analogs thereof; purified and isolated chitinase polypeptide fragments and analogs; antibodies to such polypeptides, fragments and analogs; and pharmaceutical compositions comprising these polypeptides, fragments, analogs, or antibodies.

Specifically provided are: purified, isolated polynucleotides encoding the human chitinase amino acid sequence of SEQ ID NOS: 2 or 4, particularly amino acids 1 to 445 thereof; DNAs comprising the protein coding nucleotides of SEQ ID NOS: 1 or 3, particularly nucleotides 65 to 1402 of SEQ ID NO: 1 or nucleotides 90 to 1427 of SEQ ID NO: 3; purified, isolated polynucleotides comprising a polynucleotide sequence encoding the amino acid sequence of SEQ ID NOS: 14 or 15; purified, isolated polynucleotides encoding human chitinase selected from the group consisting of: (a) a double-stranded DNA comprising the protein coding portions of the sequence set out in SEQ ID NO: 1, (b) a DNA which hybridizes under stringent conditions to a non-coding strand of the DNA of (a), and (c) a DNA which, but for the redundancy of the genetic code, would hybridize under stringent conditions to a non-coding strand of DNA sequence of (a) or (b); vectors comprising such DNAs, particularly expression vectors wherein the DNA is operatively linked to an expression control DNA sequence; host cells stably transformed or transfected with

such DNAs in a manner allowing the expression in said host cell of human chitinase; a method for producing human chitinase comprising culturing such host cells in a nutrient medium and isolating human chitinase from said host cell or said nutrient medium; purified, isolated polypeptides produced by this method; purified, isolated polypeptides comprising the human chitinase amino acid sequence of SEQ ID NOS: 2 or 4, particularly amino acids 1 to 445 thereof; human chitinase fragments lacking from 1 to about 72 C-terminal amino acid residues of mature human chitinase, particularly the human chitinase fragment of SEQ ID NO: 14; the human chitinase analog of SEQ ID NO: 15; hybridoma cell lines producing a monoclonal antibody that is specifically reactive with one of the above-described polypeptides; and monoclonal antibodies produced by such hybridomas.

Preferred DNA sequences of the invention include genomic and cDNA sequences as well as wholly or partially chemically synthesized DNA sequences. The nucleotide sequence of two human cDNAs encoding presumed allelic variants of human chitinase, and including noncoding 5' and 3' sequences, are set forth in SEQ ID NO: 1 and SEQ ID NO: 3. These DNA sequences and DNA sequences which hybridize to the noncoding strand thereof under standard stringent conditions or which would hybridize but for the redundancy of the genetic code, are contemplated by the invention. Preferred DNAs of the present invention comprise the human chitinase coding region (corresponding to nucleotides 2 to 1402 of SEQ ID NO: 1 or nucleotides 27 to 1427 of SEQ ID NO: 3), and the putative coding sequence of the mature, secreted human chitinase protein without its signal sequence (nucleotides 65 to 1402 of SEQ ID NO: 1, or nucleotides 90 to 1427 of SEQ ID NO: 3).

Exemplary stringent hybridization conditions are as follows: hybridization at 42°C in 50% formamide and washing at 60°C in 0.1 x SSC, 0.1% SDS. It is understood by those of skill in the art that variation in these conditions occurs based on the length and GC nucleotide base content of the sequences to be hybridized. Formulas standard in the art are appropriate for determining exact hybridization conditions. See Sambrook *et al.*, 9.47-9.51 in *Molecular Cloning*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1989).

Two amino acid sequences for human chitinase(s) are set forth in SEQ ID NOS: 2 and 4. The sequence of SEQ ID NO: 2 is encoded by the nucleotide sequence of SEQ ID NO: 1, and SEQ ID NO: 4 is encoded by the nucleotide sequence of SEQ ID NO: 3. Preferred polynucleotides of the present invention include, in addition to those polynucleotides described above, polynucleotides that encode amino acids -21 to 445 of SEQ ID NO: 2 or SEQ ID NO: 4, and that differ from the polynucleotides described in the preceding paragraphs only due to the well-known degeneracy of the genetic code. Similarly, since twenty-one amino acids (positions -21 to -1) of SEQ ID NOS: 2 and 4 comprise a signal peptide that is cleaved to yield the mature human chitinase protein, preferred polynucleotides include those encoding polypeptides comprising amino acids 1 to 445 of SEQ ID NO: 2 or SEQ ID NO: 4.

Among the uses for the polynucleotides of the present invention is use as a hybridization probe, to identify and isolate genomic DNA encoding human chitinase; to identify and isolate non-human genes encoding proteins homologous to human chitinase; to identify human and non-human proteins having similarity to human chitinase (including those that may be involved in tissue remodeling); and to identify those cells which express human chitinase and the biological conditions under which this protein is expressed.

In another aspect, the invention includes biological replicas (i.e., copies of isolated DNA sequences made *in vivo* or *in vitro*) of DNA sequences of the invention. Autonomously replicating recombinant constructions such as plasmid and viral DNA vectors incorporating chitinase polynucleotides, including any of the DNAs described above, are provided. Preferred vectors include expression vectors in which the incorporated chitinase-encoding cDNA is operatively linked to an endogenous or heterologous expression control sequence and a transcription terminator. Such expression vectors may further include polypeptide-encoding DNA sequences operably linked to the chitinase-encoding DNA sequences, which vectors may be expressed to yield a fusion protein comprising the polypeptide of interest.

According to another aspect of the invention, procaryotic or eucaryotic host cells are stably transformed or transfected with DNA sequences of the invention

in a manner allowing the desired chitinase product to be expressed therein. Host cells expressing chitinase products can serve a variety of useful purposes. Such cells constitute a valuable source of immunogen for the development of antibody substances specifically immunoreactive with chitinase. Host cells of the invention are useful in methods for the large scale production of chitinase wherein the cells are grown in a suitable culture medium and the desired polypeptide products are isolated, *e.g.*, by immunoaffinity purification, from the cells or from the medium in which the cells are grown.

Chitinase products may be obtained as isolates from natural cell sources or may be chemically synthesized, but are preferably produced by recombinant procedures involving procaryotic or eucaryotic host cells of the invention. Chitinase products of the invention may be full length polypeptides, fragments or analogs thereof. Chitinase products having part or all of the amino acid sequence set out in SEQ ID NO: 2 or SEQ ID NO: 4 are contemplated. One preferred fragment which lacks the C-terminal seventy-two amino acid residues of the mature protein is set forth in SEQ ID NO: 14. It has been determined that these seventy-two C-terminal residues are not critical to chitinase enzymatic activity. Example 5 illustrates production of this C-terminal fragment; the introduction of a stop codon after the codon for amino acid 373 resulted in a recombinant chitinase fragment of about 39 kDa that retained similar specific activity when compared with full length recombinant human chitinase.

Analogues may comprise chitinase analogs wherein one or more of the specified (*i.e.*, naturally encoded) amino acids is deleted or replaced or wherein one or more nonspecified amino acids are added: (1) without loss of one or more of the enzymatic activities or immunological characteristics specific to chitinase; or (2) with specific disablement of a particular biological activity of chitinase. Example 3 illustrates the production of such an analog (SEQ ID NO: 15), in which the proline at position 370 is substituted with a serine, and in which the C-terminal seventy-two amino acid residues have been deleted. The use of mammalian host cells is also expected to provide for post-translational modifications (*e.g.*, myristolation, glycosylation, truncation, lipidation and tyrosine, serine or threonine phosphorylation)

as may be needed to confer optimal biological activity on recombinant expression products of the invention.

Proteins or other molecules that bind to chitinase may be used to modulate its activity. Also comprehended by the present invention are antibody substances (e.g., monoclonal and polyclonal antibodies, single chain antibodies, chimeric antibodies, CDR-grafted antibodies and the like) and other binding proteins specific for chitinase. Proteins or other molecules (*e.g.*, small molecules) which specifically bind to chitinase can be identified using chitinase isolated from plasma, recombinant chitinase, chitinase variants or cells expressing such products. Binding proteins are useful, in turn, in compositions for immunization as well as for purifying chitinase, and are useful for detection or quantification of chitinase in fluid and tissue samples by known immunological procedures. Anti-idiotypic antibodies specific for chitinase-specific antibody substances are also contemplated.

The scientific value of the information contributed through the disclosures of DNA and amino acid sequences of the present invention is manifest. As one series of examples, knowledge of the sequence of a cDNA for chitinase makes possible the isolation by DNA/DNA hybridization of genomic DNA sequences encoding chitinase and chitinase expression control regulatory sequences such as promoters, operators and the like. DNA/DNA hybridization procedures carried out with DNA sequences of the invention under conditions of stringency standard in the art are likewise expected to allow the isolation of DNAs encoding human allelic variants of chitinase, other structurally related human proteins sharing one or more of the biochemical and/or immunological properties of chitinase, and non-human species proteins homologous to chitinase. The DNA sequence information provided by the present invention also makes possible the development, by homologous recombination or "knockout" strategies [see, *e.g.*, Kapecchi, *Science*, 244: 1288-1292 (1989)], of rodents that fail to express a functional chitinase enzyme, overexpress chitinase enzyme, or express a variant chitinase enzyme. Polynucleotides of the invention when suitably labelled are useful in hybridization assays to detect the capacity of cells to synthesize chitinase. Polynucleotides of the invention may also be the basis for diagnostic methods useful for identifying a genetic alteration(s) in the chitinase locus

that underlies a disease state or states. Also made available by the invention are anti-sense polynucleotides relevant to regulating expression of chitinase by those cells which ordinarily express the same.

Administration of chitinase preparations of the invention to mammalian subjects, especially humans, for the purpose of ameliorating disease states caused by chitin-containing parasites such as fungi is contemplated by the invention. Pathogenic fungi cause serious, often fatal disease in immunocompromised hosts. Cancer patients undergoing chemotherapy, immunosuppressed individuals, and HIV-infected individuals are susceptible to mycoses caused by *Candida*, *Aspergillus*, *Pneumocystis carinii*, and other fungi. Amphotericin B and fluconazole are useful therapeutics for fungal infections, but toxicity associated with these drugs causes serious adverse side effects that limit their usefulness. The mortality of systemic candidiasis is greater than 50% despite Amphotericin B treatment. Therefore, a need exists for agents that inhibit fungal growth *in vivo*; and such products may be used as single agents or in combination with currently approved, conventional anti-fungal compounds. Because growing fungi require chitin synthesis for survival, inhibition by recombinant human chitinase may be useful for limiting fungal infections *in vivo*. Animal models for fungal infection are illustrated below in Examples 8 through 14 and have been described in the art.

Specifically contemplated by the invention are chitinase compositions for use in methods for treating a mammal susceptible to or suffering from fungal infections comprising administering chitinase to the mammal in an amount sufficient to supplement endogenous chitinase activity. It is contemplated that the chitinase may be administered with other conventional anti-fungal agents, including amphotericin B and the structurally related compounds nystatin and pimaricin; 5-fluorocytosine; azole derivatives such as fluconazole, ketoconazole, clotrimazole, miconazole, econazole, butoconazole, oxiconazole, sulconazole, terconazole, itraconazole and tioconazole; allylamines-thiocarbamates, such as tolnaftate, naftifine and terbinafine; griseofulvin; ciclopirox olamine; haloprogin; undecylenic acid; and benzoic acid. [See, *e.g.*, Goodman & Gilman, *The Pharmacological Basis of Therapeutics*, 9th ed., McGraw-Hill, NY (1996).] Chitinase may improve the effectiveness of these conventional

anti-fungal agents, perhaps by rendering the yeast more susceptible to their action, even in situations where the chitinase alone is not effective for inhibiting growth of fungi. By reducing the amount of conventional anti-fungal agent needed to exert the desired therapeutic effect, chitinase may allow the drugs to be used at less toxic levels.

Therapeutic/pharmaceutical compositions contemplated by the invention include chitinase and a physiologically acceptable diluent or carrier and may also include other anti-fungal agents. Dosage amounts indicated would be sufficient to supplement endogenous chitinase activity. For general dosage considerations see *Remington: The Science and Practice of Pharmacy*, 19th ed., Mack Publishing Co., Easton, PA (1995). Dosages will vary between about 1 $\mu\text{g/kg}$ to 100 mg/kg body weight, and preferably between about 0.1 to about 20 mg chitinase/kg body weight. Therapeutic compositions of the invention may be administered by various routes depending on the infection to be treated, including via subcutaneous, intramuscular, intravenous, intrapulmonary, transdermal, intrathecal, topical, oral, or suppository administration.

The invention also contemplates that the overexpression of chitinase in Gaucher disease or at sites of inflammation (such as in rheumatoid arthritis) may have deleterious effects on the extracellular matrix and, in such disease settings, inhibitors of chitinase activity may provide therapeutic benefit, *e.g.* by reducing remodeling or destruction of the extracellular matrix.

The human chitinase cDNA of the present invention was isolated from a macrophage cDNA library. Macrophages are known to be closely associated with rheumatoid arthritis lesions [Feldman et al., *Cell*, 85:307-310 (1996)], and macrophage products such as TNF- α are implicated in disease progression. A protein with homology to human chitinase, C-gp39, has been detected in the synovium and cartilage of rheumatoid arthritis patients. While the natural substrate for human chitinase is probably chitin from pathogenic organisms, the enzyme may also exhibit activity on endogenous macromolecules which form the natural extracellular matrix. For example, it has been suggested that hyaluronic acid, a major component of the extracellular matrix, contains a core of chitin oligomers. [Semino et al., *Proc. Nat'l*

Acad. Sci., 93:4548-4553 (1996); Varki, *Proc. Nat'l. Acad. Sci.*, 93:4523-4525 (1996).] Chitinase may therefore be involved in degradation of extracellular matrix in diseases such as rheumatoid arthritis. The role of chitinase may be determined by measuring chitinase levels and/or the effects of chitinase administration or chitinase inhibition in synovial fluid isolated from arthritic joints. Endogenous chitinase levels can be measured by enzymatic assay or with an antibody. Viscosity of synovial fluid can be measured before and after chitinase treatment; a decrease of viscosity associated with chitinase would be consistent with an endogenous chitinase substrate. Modulation of chitinase activity could thereby modulate the progression of joint destruction in rheumatoid arthritis.

Also contemplated by the invention are methods for screening for inhibitors of chitinase activity, which may be useful in the manner described in the preceding paragraph. A method for screening samples to identify agents that inhibit chitinase is reported in, *e.g.*, WO 95/34678 published 21 December 1995.

Further contemplated are methods for measuring endogenous levels of chitinase, *e.g.*, for diagnosing Gaucher's disease. Hollak et al., *J. Clin. Invest.*, 93:1288-1292 (1994), report that plasma chitinase levels are a diagnostic marker for Gaucher's disease. The recombinant proteins of this invention are expected to be more useful than preparations purified from humans, which have associated problems of yield and contamination with other impurities or infectious agents.

DETAILED DESCRIPTION

Other aspects and advantages of the present invention will be understood upon consideration of the following illustrative examples. Example 1 describes the isolation of human chitinase cDNA clones from a human macrophage cDNA library. Example 2 addresses the pattern of chitinase gene expression in various human tissues. Example 3 describes the recombinant expression of the human chitinase gene in prokaryotic cells and purification of the resulting enzyme. Example 4 provides a protocol for the recombinant production of human chitinase in yeast. Example 5 describes the recombinant expression of the human chitinase gene in mammalian cells and purification of the resulting protein. Example 6 describes

production of human chitinase polypeptide analogs by peptide synthesis or recombinant production methods. Example 7 provides a protocol for generating monoclonal antibodies that are specifically immunoreactive with human chitinase. Example 8 describes an assay for the measurement of chitinase catalytic activity. Example 9 addresses determination of the anti-fungal activity of human chitinase *in vitro*. Example 10 addresses determination of the anti-fungal activity of human chitinase *in vivo* in a mouse model, and Examples 11 through 14 address rabbit models of invasive aspergillosis, disseminated candidiasis, *Candida* ophthalmitis, and *Candida* endocarditis.

Example 1

Isolation of Chitinase cDNA Clones

A cDNA library was prepared from peripheral blood monocyte-derived macrophages as described in Tjoelker et al., *Nature*, 374:549-552 (1995). Clones from the library were randomly chosen and plasmid DNA was purified from individual clones. The sequence of approximately 300 to 500 bases from the end of DNA from each clone was determined on an automated sequencer (Model 373, Applied Biosystems, Foster City, CA) using primer JHSP6, which hybridizes to the plasmid vector pRc/CMV (Invitrogen, San Diego, CA) adjacent to the cDNA cloning site:

JHSP6: 5'-GACACTATAGAATAGGGC-3' (SEQ ID NO: 5)

The nucleotide and deduced amino acid sequence of these cDNA clones were compared to sequences in nucleotide and peptide sequence databases to determine similarity to known genes. Sequence comparisons were performed by the BLAST Network Service of the National Center for Biotechnology Information using the alignment algorithm of Altschul et al., *J. Mol. Biol.*, 215:403-410 (1990). Clone MO-911 exhibited significant homology to several different sequences, including mouse macrophage secretory protein YM-1 precursor (Genbank accession no. M94584), human cartilage gp-39 (Hakala *et al.*, *supra*), oviductal glycoprotein from sheep, cow, and humans (DeSouza *et al.*, *supra*), and chitinases from parasite (*Oncocerca*, Genbank accession no. U14639), wasp (*Chelonus*, Genbank accession no.

U10422), plant (*Nicotiana*, Genbank accession no. X77111), and bacteria (*Serratia*, Genbank accession no. Z36295); its highest observed homology was to mammalian genes that encoded proteins with chitinase homology but no demonstrated chitinase activity. Further sequence analysis of MO-911 suggested that it contained a portion of the coding region for a human chitinase homolog.

The DNA sequence of clone pMO-218 (deposited on June 7, 1996 under the terms of the Budapest Treaty with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852, U.S.A. under Accession No. 98077) is set forth in SEQ ID NO: 1, and the encoded amino acid sequence is set forth in SEQ ID NO: 2. MO-218 appeared to include the entire coding region of the human chitinase cDNA (nucleotides 2 to 1402 of SEQ ID NO: 1), which comprises a twenty-one amino acid putative signal sequence followed by 445 encoded amino acids (residues 1 to 445 of SEQ ID NO: 2). The twenty-two amino acids following the putative signal sequence exactly match the amino-terminal sequence of purified human chitotriosidase reported in Renkema *et al.*, *supra*. Renkema *et al.* also described a twenty-one amino acid sequence from a tryptic fragment of human chitotriosidase which corresponds exactly to residues 157 to 177 of MO-218 (SEQ ID NO: 2). Boot *et al.*, *supra*, report the cloning of a human chitotriosidase cDNA which contains a coding sequence essentially identical to that of MO-218. The sequence of MO-218 differs from Boot *et al.* by an additional fourteen nucleotides at the 5' end and by a nucleotide change at nucleotide 330 in the coding region.

To confirm that MO-218 indeed contained the entire coding region of the cDNA, a ³²P-labelled probe P-1 (TGGGATCATCAGCAGGACCATGAAACCTGCCCAGGCCACAGACCGCACCAT, SEQ ID NO: 6) was prepared that corresponded to the complement of nucleotides 2 through 52 of MO-218 (SEQ ID NO: 1). Probe P-1 was designed to hybridize with clones that are at least as long as MO-218 at the 5' end. The probe was hybridized with a portion (approximately 30,000 clones) of the human macrophage cDNA library described above, in 40% formamide and hybridization buffer (5 x SSPE, 10 x Denhardt's, 100 µg/ml denatured salmon sperm DNA, and 2% SDS) at 42°C overnight. The filters were washed and three clones that hybridized were chosen for

sequence /analysis. The longest clone was designated pMO-13B (deposited on June 7, 1996 under the terms of the Budapest Treaty with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852, U.S.A. under Accession No. 98078). The DNA sequence of pMO-13B is set forth in SEQ ID NO: 3 and the encoded amino acid sequence is set forth in SEQ ID NO: 4. This clone contains 25 additional nucleotides at the 5' end compared with MO-218; in addition, MO-13B (SEQ ID NO: 3) contains one nucleotide substitution at nucleotide 330 (corresponding to nucleotide 305 of MO-218, SEQ ID NO: 1) which changes the encoded amino acid at position 80 of the mature protein from a glycine (in SEQ ID NO: 2) to a serine (in SEQ ID NO: 4).

Example 2

Chitinase Gene Expression Pattern in Human Tissues

Northern blot analysis was performed to identify tissues in which the human chitinase is expressed. A multiple human tissue Northern blot (Clontech, Palo Alto CA) was hybridized with the entire coding region of MO-218 under standard stringent conditions (according to the Clontech laboratory manual). Greatest hybridization was observed to lung tissue (+++) and ovary (+++), with much smaller levels (+) in thymus and placenta. The size of the hybridizing mRNA was 2.0 kb for lung, ovary and thymus, which corresponds well with the size of the cloned cDNA (1.6 kb, or about 1.8 kb including the polyA tail). The size of the hybridizing placental mRNA was considerably smaller, at 1.3 kb. Chitinase hybridization was not observed in spleen, prostate, testes, small intestine, colon, peripheral blood leukocytes, heart, brain, liver, skeletal muscle, kidney, or pancreas. Chitinase expression in lung is consistent with a protective role against pathogenic organisms that contain chitin, since the lung represents the primary route of entry for fungal pathogens.

Example 3

Production of Recombinant Human Chitinase in Bacterial Cells

The mature coding region of MO-218 was engineered for expression in *E. coli* as a C-terminal truncated analog. PCR was used to generate a DNA fragment for expression using a primer corresponding to nucleotides 65 to 88 of the MO-218 chitinase cDNA preceded by an initiating methionine codon and an XbaI restriction endonuclease site (5'-

TACATCTAGAATTATGGCAAACTGGTCTGCTACTTCACC-3', SEQ ID NO: 7), and a downstream primer encoding nucleotides 1163 to 1183 of MO-218 followed by a stop codon and a HindIII site (5'-

AGATCTAACCTTAGGTGCCTGAAGACAAGTATGG-3', SEQ ID NO: 8). The downstream primer contained an adenine at base 25, while the MO-218 sequence contains a guanine at the corresponding nucleotide position. Consequently, the resulting DNA fragment contains a thymine rather than a cytosine at the position corresponding to nucleotide 1172 of the MO-218 sequence, and the encoded chitinase fragment, set forth in SEQ ID NO: 15, is also an analog that contains a serine at mature amino acid position 370 instead of the proline encoded by MO-218. The resulting DNA fragment was digested with XbaI and HindIII and cloned into plasmid pAraBAD (which is also known by the designation pAraCB).

Plasmid pAraCB was prepared as follows. Plasmid pUC19 was modified to include an arabinose promoter and subsequently to include AKAP 79 encoding sequences. The arabinose promoter [Wilcox *et al.*, *Gene* 34:123-128 (1985); Wilcox, *et al.*, *Gene* 18:157-163 (1982)] and the *araC* gene were amplified by PCR from the arabinose operon BAD of *Salmonella typhimurium* as an *EcoRI/XbaI* fragment with the primers *araC*-2 (SEQ ID NO: 9) and *arab*-1 (SEQ ID NO: 10):

araC-2 TACAGAATTCTTATTCACATCCGGCCCTG SEQ ID NO: 9

arab-1 TACATCTAGACTCCATCCAGAAAAACAGGTATGG SEQ ID NO: 10

Primer *araC*-2 encodes an *EcoRI* site (underlined) and a termination codon (italics) for the *araC* gene product. Primer *arab*-1 encodes a putative ribosome binding domain

(italics) and an *Xba*I restriction site (underlined). PCR with these primers produced a 1.2 kb fragment which was digested with *Eco*RI and *Xba*I and subcloned into pUC19 (New England Biolabs, Beverly, MA) previously digested with the same two enzymes. The resulting plasmid was designated araCB and contained a polylinker region (SEQ ID NO: 11) flanked at the 5' end with a *Xba*I restriction site (underlined) and at the 3' end with a *Hind*III site (italics).

araCB polylinker

SEQ ID NO: 11

TCTAGAGTCGACCTGCAGGCATGCAAGCTT

Transformants containing the resulting expression plasmid (pAraMO218) were induced with arabinose and grown at 37°C. These transformants produced inclusion bodies containing a 39 kDa protein which was a truncated form of chitinase (engineered to contain 373 instead of 445 amino acids). This chitinase fragment contains four cysteine residues, while the full length chitinase contains ten cysteine residues. The inclusion bodies were separated from the *E. coli* culture and electrophoresed on SDS-PAGE. The 39 kDa band was transferred to a PVDF membrane and amino terminal sequenced. The majority (about two-thirds) of the material contained a sequence corresponding to the amino terminus of human chitinase. The remaining material corresponded to a contaminating *E. coli* protein, porin. This recombinant chitinase preparation from *E. coli* was useful for producing polyclonal and monoclonal antibodies (described below in Example 7).

When transformants containing the Ara-chitinase expression plasmid were grown at 25°C, inclusion bodies were not observed and expression of recombinant product was decreased from about ten percent of total cell protein to about one percent. However, this material produced at 25°C exhibited chitinase catalytic activity.

origin of replication. The beta-lactamase and TRP1 genes provide for selection in bacteria and yeast, respectively. The REP1 and REP2 genes encode proteins involved in plasmid copy number replication.

Alternatively, other fusion points within the chitinase coding region may be chosen. Truncates of the coding region may be used to increase homogeneity of the product, increase the specific activity or alter the substrate specificity.

The DNA constructs described in the preceding paragraphs are transformed into yeast cells using a known method, *e.g.* lithium acetate treatment [Stearns *et al.*, *Meth. Enz.*, *supra*, pp. 280-297] or by equivalent methods. The ADH2 promoter is induced upon exhaustion of glucose in the growth media [Price *et al.*, *Gene*, 55:287 (1987)]. The pre-pro-alpha sequence or other leader sequence effects secretion of the fusion protein, releasing the mature human chitinase peptide from the cells. The signal peptide leader is processed by signal peptidase or, in the case of pre-pro-alpha removal of the pro region, by the KEX2 protease [Bitter *et al.*, *Proc. Natl. Acad. Sci. USA*, 81:5330-5334 (1984)].

Chitinase contains in its mature amino acid sequence two dibasic sequences at positions 107-108 (Lys-Arg) and 209-210 (Arg-Lys) that may be proteolytically clipped by the KEX2 protease during secretion. To stabilize and/or increase the level of product secreted from cells, these sequences could be mutated to eliminate the potential sites for proteolysis as shown by Gillis *et al.* [*Behring Inst. Mitt.*, No. 83:1-7 (1988)] or by expressing chitinase without dibasic modifications in a host that is deficient in KEX2. Such hosts can be obtained either by screening for non-KEX2 protease containing mutants, or by manipulation of the genomic KEX2

locus by gene replacement/gene disruption techniques [Orr-Weaver *et al.*, *Proc. Natl. Acad. Sci, USA*, 78:6354-6358 (1981)].

Recombinant chitinase may be secreted from *Saccharomyces cerevisiae* using similar vectors containing alternative promoters PRB1, GAL4, TPI, or other suitably strong promoters bearing fragments or by fusion to a variety of leader sequences [Sleep *et al.*, *Bio/Technol.*, 8:42-46 (1990)].

Other non-*Saccharomyces cerevisiae* suitable expression hosts include *Kluyveromyces lactis*, *Schizosaccharomyces pombe*, *Pichia pastoris* and members of the *Hansenula* or *Aspergillus* geni. Analogous recombinant expression systems for these fungi include the organism and their appropriate autonomously replicating vector [e.g. Falcone *et al.*, *Plasmid*, 15:248-252 (1988)] or multiply integrated expression cassettes. These systems also rely on signal sequences or leaders of the types described above to mediate secretion into the medium.

The secreted recombinant human chitinase is purified from the yeast growth medium by, e.g., the methods used to purify chitinase from bacterial and mammalian cell supernatants (see Example 3 above and Example 5 below).

Alternatively, the mature form of the recombinant chitinase product may be expressed in the cytoplasm of the *Saccharomyces cerevisiae* cells or analogous host, and purified from the lysed host cells. The protein may be refolded during the act of purification to obtain appropriate levels of specific activity.

Example 5

Production of Recombinant Human Chitinase in Mammalian cells

The MO-218 clone and the MO-13B clone, both of which contain full length human chitinase cDNA 3' to the CMV promoter of pRc/CMV, were isolated.

5 A third plasmid, which corresponded to the same C-terminal fragment expressed in bacterial cells in Example 3 above, was prepared as follows. The MO-218 plasmid was amplified by PCR using oligonucleotide primer 218-1

(CGCAAGCTTGAGAGCTCCGTTCCGCCACATGGTGCGGTCTGTGGCCTGGG, SEQ ID NO: 12), which contains a Hind III site and nucleotides 2 through 23 of the

10 MO-218 chitinase cDNA of SEQ ID NO: 1, and with complementary downstream primer T-END (GACTCTAGACTAGGTGCCTGAAGGCAAGTATG, SEQ ID NO: 13), which contains nucleotides 1164 through 1183 of MO-218, a stop codon and an

XbaI site. The amplified DNA was purified by electrophoresis, digested with XbaI and HindIII, and cloned into the pRc/CMV vector (Invitrogen, San Diego, CA)

15 previously cut with the same restriction enzymes. The junctions of the resulting clone was sequenced on a Model 373 (Applied Biosystems, Foster City, CA) and encoded the predicted engineered protein sequence, set forth in SEQ ID NO: 14.

All three plasmids were transiently transfected into COS cells by the DEAE transfection method [see, *e.g.*, Sambrook *et al.*, *Molecular Cloning: a*

20 *Laboratory Manual*, 2d ed., Cold Spring Harbor, New York: Cold Spring Harbor Laboratory (1989).). After three days at 37°C, media from cells was assayed for chitinase activity *in vitro* as described below in Example 8. Each culture produced significant chitinase activity (600-800 mU/ml/min), and similar amounts were observed for each construct.

Recombinant human chitinase was purified as follows. Five days after transfection of COS cells with the pRc/CMV-MO-13B plasmid, conditioned media from the culture was harvested and diluted with an equal volume of water. The diluted conditioned media was applied to a Q-Sepharose Fast Flow column (Pharmacia Biotech, Uppsala, Sweden) which was pre-equilibrated in 25 mM Tris, 10 mM sodium chloride, 1 mM EDTA, at pH 8.0. Approximately 95 % of the chitinase activity flowed through and did not bind to the column. This Q-Sepharose flow through was adjusted to 1.2 M ammonium sulfate and applied to a Butyl-Sepharose 4 Fast Flow column (Pharmacia) which was pre-equilibrated in 25 mM Tris, 1.2 M ammonium sulfate, 1 mM EDTA, at pH 8.0. Protein was eluted using a reverse gradient of 1.2 M to 0 M ammonium sulfate in 25 mM Tris, pH 8.0. A single absorbance peak at 280nm corresponding to the chitinase activity peak was eluted at low salt. This material was greater than 85 % pure as determined by SDS-PAGE and contained approximately 60% of the chitinase activity. The protein was then concentrated and buffer exchanged into 20 mM Tris, 150 mM sodium chloride, at pH 8.0 using a 10,000 MWCO membrane (Ultrafree 10K, Millipore Corp., Bedford, MA). This preparation was then tested for enzymatic and anti-fungal activity *in vitro* as described in Examples 8 and 9 below. The recombinant preparation had a chitotriosidase activity of 90 nm/min per mg protein.

Example 6

Production of Human Chitinase Analogs and Fragments

Recombinant techniques such as those described in the preceding examples may be used to prepare human chitinase polypeptide analogs or fragments.

5 More particularly, polynucleotides encoding human chitinase are modified to encode polypeptide analogs of interest using well-known techniques, *e.g.*, site-directed mutagenesis and polymerase chain reaction. C-terminal and N-terminal deletions may also be prepared by, *e.g.*, deleting the appropriate portion of the polynucleotide coding sequence. See generally Sambrook *et al.*, *supra*, Chapter 15. The modified
10 polynucleotides are expressed recombinantly, and the recombinant polypeptide analogs or fragments are purified as described in the preceding examples.

Residues critical for human chitinase activity are identified, *e.g.*, by homology to other chitinases and by substituting alanines for the native human chitinase amino acid residues. Cysteines are often critical for the functional integrity
15 of proteins because of their capacity to form disulfide bonds and restrict secondary structure. To determine whether any of the cysteines in human chitinase are critical for enzymatic activity, each cysteine is mutated individually to a serine.

A 39 kDa C-terminally truncated fragment of the mature human chitinase protein was prepared as described above in Examples 3 and 5 by
20 introduction of a stop codon after the codon for amino acid 373. This 39 kDa fragment lacked seventy-two C-terminal amino acid residues of the mature protein, including six cysteines, yet retained similar specific enzymatic activity compared to the full length recombinant human chitinase. This result indicates that the missing

seventy-two C-terminal residues, including the six cysteines, are not required for enzymatic activity.

Further C-terminal deletions may be prepared, *e.g.*, by digesting the 3' end of the truncated human chitinase coding sequence described in Example 3 with exonuclease III for various amounts of time and then ligating the shortened coding sequence to plasmid DNA encoding stop codons in all three reading frames. N-terminal deletions are prepared in a similar manner by digesting the 5' end of the coding sequence and then ligating the digested fragments into a plasmid containing a promoter sequence and an initiating methionine immediately upstream of the promoter site. These N-terminal deletion analogs or fragments may also be expressed as fusion proteins.

Alternatively, human chitinase polypeptide analogs may also be prepared by full or partial chemical peptide synthesis using techniques known in the art. [See, *e.g.*, synthesis of IL-8 in Clark-Lewis *et al.*, *J. Biol Chem.*, 266:23128-34 (1991); synthesis of IL-3 in Clarke-Lewis *et al.*, *Science*, 231:134-139 (1986); and synthesis by ligation in Dawson *et al.*, *Science*, 266:776-779 (1994).] Such synthetic methods also allow the selective introduction of novel, unnatural amino acids and other chemical modifications.

The biological activities, including enzymatic, anti-fungal, and extracellular matrix remodeling activities, of the human chitinase polypeptide analogs are assayed by art-recognized techniques, such as those described in Examples 8 to 14 below.

Example 7

Preparation of Monoclonal Antibodies to Human Chitinase

The following two protocols (multiple challenge or single shot immunizations) may be used to generate monoclonal antibodies to human chitinase.

5 In the first protocol, a mouse is injected periodically with recombinant human chitinase (*e.g.*, 10-20 μ g emulsified in Freund's Complete Adjuvant) obtained as described in any of Examples 3 through 6. The mouse is given a final pre-fusion boost of human chitinase in PBS, and four days later the mouse is sacrificed and its spleen removed. The spleen is placed in 10 ml serum-free RPMI 1640, and a single
10 cell suspension is formed by grinding the spleen between the frosted ends of two glass microscope slides submerged in serum-free RPMI 1640, supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 100 units/ml penicillin, and 100 μ g/ml streptomycin (RPMI) (Gibco, Canada). The cell suspension is filtered through sterile 70-mesh Nitex cell strainer (Becton Dickinson, Parsippany, New Jersey), and is
15 washed twice by centrifuging at 200 g for 5 minutes and resuspending the pellet in 20 ml serum-free RPMI. Splenocytes taken from three naive Balb/c mice are prepared in a similar manner and used as a control. NS-1 myeloma cells, kept in log phase in RPMI with 11% fetal bovine serum (FBS) (Hyclone Laboratories, Inc., Logan, Utah) for three days prior to fusion, are centrifuged at 200 g for 5 minutes, and the pellet is
20 washed twice as described in the foregoing paragraph.

One $\times 10^8$ spleen cells are combined with 2.0×10^7 NS-1 cells and centrifuged, and the supernatant is aspirated. The cell pellet is dislodged by tapping the tube, and 1 ml of 37°C PEG 1500 (50% in 75mM Hepes, pH 8.0) (Boehringer Mannheim) is added with stirring over the course of 1 minute, followed by the

addition of 7 ml of serum-free RPMI over 7 minutes. An additional 8 ml RPMI is added and the cells are centrifuged at 200 g for 10 minutes. After discarding the supernatant, the pellet is resuspended in 200 ml RPMI containing 15% FBS, 100 μ M sodium hypoxanthine, 0.4 μ M aminopterin, 16 μ M thymidine (HAT) (Gibco), 25 units/ml IL-6 (Boehringer Mannheim) and 1.5×10^6 splenocytes/ml and plated into 10 Corning flat-bottom 96-well tissue culture plates (Corning, Corning New York).

On days 2, 4, and 6, after the fusion, 100 μ l of medium is removed from the wells of the fusion plates and replaced with fresh medium. On day 8, the fusion is screened by ELISA, testing for the presence of mouse IgG binding to human chitinase as follows. Immulon 4 plates (Dynatech, Cambridge, MA) are coated for 2 hours at 37°C with 100 ng/well of human chitinase diluted in 25mM Tris, pH 7.5. The coating solution is aspirated and 200 μ l/well of blocking solution [0.5% fish skin gelatin (Sigma) diluted in CMF-PBS] is added and incubated for 30 min. at 37°C. Plates are washed three times with PBS with 0.05% Tween 20 (PBST) and 50 μ l culture supernatant is added. After incubation at 37°C for 30 minutes, and washing as above, 50 μ l of horseradish peroxidase conjugated goat anti-mouse IgG(fc) (Jackson ImmunoResearch, West Grove, Pennsylvania) diluted 1:3500 in PBST is added. Plates are incubated as above, washed four times with PBST, and 100 μ L substrate, consisting of 1 mg/ml o-phenylene diamine (Sigma) and 0.1 μ l/ml 30% H₂O₂ in 100 mM Citrate, pH 4.5, are added. The color reaction is stopped after 5 minutes with the addition of 50 μ l of 15% H₂SO₄. A₄₉₀ is read on a plate reader (Dynatech). Selected fusion wells are cloned twice by dilution into 96-well plates and visual scoring of the number of colonies/well after 5 days. The monoclonal

antibodies produced by hybridomas are isotyped using the Isostrip system (Boehringer Mannheim, Indianapolis, IN).

Alternatively, a second protocol utilizing a single-shot intrasplenic immunization may be conducted generally according to Spitz, *Methods Enzymol.*, 121:33-41 (1986). The spleen of the animal is exposed and injected with recombinant human chitinase (*e.g.*, 10-20 μ g in PBS at a concentration of about 0.02 % to 0.04 %, with or without an aluminum adjuvant) obtained as described in any of Examples 3 through 6, after which the spleen is returned to the peritoneal cavity and the animal is stitched closed. Three days later, the mouse is sacrificed and its spleen removed. A spleen cell suspension is prepared, washed twice with RPMI 1640 supplemented with 3 % fetal calf serum (FCS), and resuspended in 25 ml of the same medium. Myeloma cells (NS-O) are collected at logarithmic growth phase, washed once and added to the spleen cell suspension in a 50 ml tube, at a ratio of 3:1 or 2:1 (spleen cells:myeloma cells). The mixture is pelleted at about 450 g (1500 rpm), the supernatant aspirated, and the pellet loosened by tapping the tube. Fusion is performed at room temperature by adding 1 ml of polyethylene glycol (PEG) 1500 over 1 minute, with constant stirring. The mixture is incubated for another minute, then 1 ml of warm RPMI (30 to 37°C) is added over 1 minute followed by 5 ml RPMI over 3 minutes and another 10 ml RPMI over another 3 minutes. The cell suspension is centrifuged and resuspended in about 200 ml of HAT selective medium consisting of RPMI 1640 supplemented with 100 U/ml penicillin, 100 μ g/ml streptomycin, 20 % FCS, 100 mM hypoxanthine, 0.4 mM aminopterin and 16 mM thymidine. The cell suspension is dispensed in 1 ml volumes into tissue culture plates and incubated at 37°C in a humid atmosphere with 5 % CO₂-95 % air for 8 to 10 days. Supernatants are aspirated and

the cells are fed with 1 ml HAT medium per well, every 2 to 3 days according to cell growth. Supernatants of confluent wells are screened for specific antibodies and positive wells are cloned.

Example 8

Catalytic Activity of Recombinant Chitinase

Chitotriosidase (chitinase) activity was measured using the fluorogenic substrate 4-methylumbelliferyl- β -D-N,N',N''-triacetylchitotriose (4 MU-chitotrioside, Sigma Chemical, St. Louis, MO) in McIlvain buffer (Hollak *et al.*, *supra*). Ten μ l samples of the recombinant product were combined with 10 μ l bovine serum albumin (10 mg/ml), 15 μ l fluorogenic substrate (2.71 mM), and 65 μ l buffer (0.1M citric acid, 0.2M sodium phosphate, pH 5.2) in a total volume of 100 μ l. Reactions were incubated at 37°C for 15 minutes, then the reaction was stopped with the addition of 2 ml of 0.3M glycine/NaOH buffer (pH 10.6). The fluorescent cleavage product, 4-methylumbelliferone, was monitored with a fluorimeter (SLM-AMINCO Instruments, Inc., Rochester, NY) at 450 nm. To obtain a standard curve, several substrate concentrations were combined with excess bacterial chitinase to ensure that substrate was completely cleaved. The known quantity of 4-MU was then correlated to the fluorescence signal from the fluorimeter and linear regression was used to determine a standard curve. The signal produced with diluted purified recombinant chitinase in the assay was then used to interpolate the nm quantity of substrate cleaved by the enzyme during the reaction time. This number was then divided by the concentration of protein to obtain the nm/min per mg protein (determined by A₂₈₀ and calculated molar extinction coefficient).

The chitotriosidase activity of the recombinant human chitinase from Example 5 was determined to be 90 nm/min per mg protein.

Example 9

Anti-fungal Activity of Recombinant Chitinase *In Vitro*

5 In a preliminary experiment, recombinant human chitinase was tested for inhibition of fungal growth *in vitro*. The two fungi *Candida albicans* and *Aspergillus fumigatis* are serious pathogens for immunocompromised patients. Both *Candida* and *Aspergillus* were grown in RPMI growth media at 37°C to approximately 10,000-50,000 colony forming units (CFU) per ml. Recombinant human chitinase (prepared as described in Example 5) was added to cultures at 0, 2.8, 11.25, or 45 µg/ml. After 24 hours, fungal growth was assessed by turbidity of cultures. Under these non-physiological conditions in this assay, all cultures appeared to grow at comparable rates, independent of chitinase concentration. The concentration of fungi tested, however, is much higher than the fungal burden seen during fungal infection *in vivo*. Different results may be obtained under different conditions, *e.g.*, with a lower fungal burden, or when human chitinase is tested in combination with other anti-fungal agents. Chitinase is also expected to be more effective *in vivo* under physiological conditions.

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Example 10

Anti-fungal Activity of Recombinant Chitinase *In Vivo* in Mice

Several animal models have been developed for testing efficacy of anti-fungal compounds [see Louie et al., *Infect. Immun.*, 62: 2761-2772, 1994; Kinsman et al., *Antimicrobial Agents and Chemotherapy*, 37: 1243-1246, 1993; Nakajima et al., *Antimicrobial Agents and Chemotherapy* 39: 1517-1521, 1995; Tonetti et al., *Eur. J. Immunol.*, 25:1559-1565 (1995)]. Briefly, the animal host is infected with the fungi, varying doses of chitinase are administered to the animals, and their survival is measured over time. The experiments are performed using chitinase as the sole therapeutic agent, or with a combination of conventional anti-fungal agents such as Amphotericin B and fluconazole to determine if the chitinase improves the efficacy of such compounds. Specifically, acute systemic candidiasis is achieved in mice by intraperitoneal or intravenous challenge of 10×10^6 CFU *Candida albicans*. The therapeutic agents are administered before or at 1 to 5 hours after challenge, and the number of survivors is determined after five days. In addition, the mice can be sacrificed and fungal load can be determined in specific organs such as kidney, lung, liver and spleen. Alternatively, the mice are challenged with lower doses of *Candida* (1×10^6 CFU), in which case survival can be measured at more distant time points, e.g., 45 days. Effective anti-fungal agents enhance the long term survival of animals and reduce fungal load in blood and organs.

Example 11

Activity of Chitinase *In Vivo* in a Rabbit Model of Invasive Aspergillosis

The efficacy of chitinase, alone or in combination with other conventional anti-fungal agents, is assessed in an immunosuppressed rabbit model of invasive aspergillosis which has been used for over ten years to evaluate a variety of anti-fungal therapies. See, *e.g.*, Andriole et al., *Clin. Infect. Dis.*, 14(Suppl. 1):S134-S138 (1992). The study is conducted generally according to Patterson et al., *Antimicrob. Agents Chemother.*, 37:2307-2310 (1993) or George et al., *J. Infect. Dis.*, 168:692-698 (1993). Briefly, on day one the rabbits are given cyclophosphamide (200 mg) intravenously to render them leukopenic, followed by triamcinolone acetonide (10 mg) subcutaneously each day for the duration of the experiment. On day two, 24 hours after immunosuppression, the animals are challenged intravenously with about 10^6 (lethal challenge) or about 10^5 (sublethal challenge) *A. fumigatus* conidia. Anti-fungal therapy (chitinase alone, or in combination with other conventional anti-fungal agents, *e.g.*, amphotericin B, fluconazole, or 5-fluorocytosine) is initiated at 24 hours after challenge or 48 hours before challenge (for prophylaxis) and is continued for 5 to 6 days or until death. Exemplary doses of conventional anti-fungal agents are 1.5 or 0.5 mg/kg/day intravenous amphotericin B, 60 or 120 mg/kg/day oral fluconazole and 100 mg/kg/day oral 5-fluorocytosine. Control rabbits are not treated with any anti-fungal agent.

At autopsy or death, semiquantitative fungal cultures and histopathologic examination are conducted on the liver, spleen, kidneys, lungs and brain. Cultures of the heart, urine and blood may also be performed. Blood samples

are obtained at intervals and assayed for white blood cell counts and circulating *Aspergillus* carbohydrate antigen using an ELISA assay. The effect of treatment with the test drug is evaluated on three endpoints: reduction in mortality rate, reduction in number of *Aspergillus* organisms cultured from target organs (fungal burden), and reduction in level of circulating *Aspergillus* antigen. Effective anti-fungal agents reduce mortality and/or fungal load.

Alternatively, pulmonary aspergillosis may be evaluated in this model generally according to Chilvers et al., *Mycopathologia*, 108:163-71 (1989), in which the immunosuppressed rabbits are challenged with intratracheal instillation of *Aspergillus fumigatus* conidia, followed by bronchoalveolar lavage on days 1, 2, 4, 7 and 10 following challenge; fungal culture, chitin assay, white cell counts and histopathology are performed on the lavage fluids to determine infective load within the lung. Effective fungal agents reduce the infective load or inflammation within the lung.

Example 12

Activity of Chitinase *In Vivo* in a Rabbit Model of Disseminated Candidiasis

The efficacy of chitinase, alone or in combination with other conventional anti-fungal agents, is assessed in a rabbit model of disseminated candidiasis generally according to Rouse et al., *Antimicrob. Agents Chemother.*, 36:56-58 (1992). New Zealand white rabbits are infected systemically with about 3×10^6 *Candida albicans* blastospores. Anti-fungal therapy is initiated 48 hours after challenge with *Candida* (or before challenge for prophylaxis) and is continued for,

e.g., four days. Surviving animals are sacrificed, and fungal cultures are performed on the aortic valve with attached vegetation, lung, kidney and spleen. Fungal cultures and histopathological examination may also be performed on these and other organs, such as liver, brain, and heart. Urine and blood cultures may also be done. The effect of the anti-fungal therapy on mortality and circulating or tissue fungal burden is determined.

Bayer et al., *Antimicrob. Agents Chemother.*, 19:179-184 (1981), in which rabbits are inoculated intraperitoneally with about 5×10^8 CFU *Candida albicans*. A saline peritoneal aspirate is obtained and cultured from each animal four days after intraperitoneal inoculation, and animals with a positive fungal culture aspirate are randomly assigned to control or treatment groups. Anti-fungal treatment is begun seven days after challenge. The eyes of all rabbits are evaluated using indirect ophthalmoscopy, as disseminated candidiasis may result in *Candida* endophthalmitis. Animals are sacrificed at 7, 11 and 14 days after initiation of therapy and their abdomens inspected for evidence of peritonitis and intraabdominal abscess formation. Eyes are examined for macroscopic lesions. Tissue samples from peritoneal abscesses, all other visible abscesses, kidneys, livers, spleens and ocular structures are weighed, homogenized in brain heart infusion broth, serially diluted and cultured to determine the CFU per gram of tissue. Renal and peritoneal abscesses are also fixed in 10% neutral formaldehyde and examined for histopathology. Sections are stained with periodic acid-Schiff reagent to determine the fungal burden and fungal morphology. Effect of the test drugs on improving survival and reducing fungal burden is evaluated.

Example 13

Activity of Chitinase *In Vivo* in a Rabbit Model of Fungal Endophthalmitis

The efficacy of chitinase, alone or in combination with other conventional anti-fungal agents, is assessed in a rabbit model of *Candida* endophthalmitis, generally according to Park et al., *Antimicrob. Agents Chemother.*, 39:958-963 (1995). Briefly, New Zealand albino rabbits, 2 to 2.5 kg, are infected with an intravitreal inoculation of about 1,000 CFU of *Candida albicans*. Endophthalmitis is confirmed 5 days after inoculation by indirect ophthalmoscopy, and is defined as moderate to severe vitreous haze with partial or complete obscuration of greater than 50% of the retinal and choroidal vasculature. The vitreous turbidity is graded on a scale, and the fundus appearance may be graded and documented by fundus photography. The rabbits are then randomized to the following treatment conditions: chitinase alone for 2 to 4 weeks, a combination of chitinase and another conventional anti-fungal agent (*e.g.*, amphotericin B, fluconazole or 5-fluorocytosine) for 2 to 4 weeks, or no treatment (control). Exemplary doses of conventional anti-fungal agents are 80 mg/kg/day of oral fluconazole and 100 mg/kg every 12 hours of oral 5-fluorocytosine.

The treatment effect is assessed at 2 and 4 weeks after therapy by indirect ophthalmoscopy, quantitative fungal culture, and histopathology. For quantitative fungal culture, the eyes are dissected and weighed, and a weighed fraction of each sample is homogenized and cultured on brucella agar-5% horse blood plates for 48 hours at 35°C in 5 to 10% CO₂. The homogenized sample may also be diluted 10- or 100-fold with sterile saline before plating. The colonies are counted

and the total CFU in the eye calculated on the basis of the growth yielded from the measured fractions of sample. Treatment effect is assessed in terms of a reduction in the total intraocular fungal burden. For histopathology, representative eyes are removed, fixed in formalin, embedded in plastic, and sliced into 5 μ m sections. The sections are stained with hematoxylin-eosin or Gomori's methenamine silver stain and examined by light microscopy for inflammation, fibrous organization and fungal elements. The effect of the anti-fungal agents on reducing mortality, reducing fungal load, or reducing the inflammation associated with fungal infection, is evaluated.

Alternatively, a rabbit model of *Aspergillus* endophthalmitis may be used generally according to Jain et al., *Doc. Ophthalmol.*, 69:227-235 (1988). Briefly, New Zealand white rabbits are inoculated in one eye with about forty spores of *Aspergillus fumigatus*. Their contralateral (control) eyes receive a similar but sterile inoculum. After treatment with the test drug (chitinase alone, or chitinase in combination with another agent), the rabbits' eyes may be evaluated for clinical appearance, electroretinogram waveforms, indirect ophthalmoscopy, quantitative fungal culture, and histopathology. Clinically evident endophthalmitis typically develops within three to seven days after inoculation.

Example 14

Activity of Chitinase *In Vivo* in a Rabbit Model of Fungal Endocarditis

The efficacy of chitinase, alone or in combination with other conventional anti-fungal agents, is assessed in a rabbit model of *Candida* endocarditis generally according to Witt and Bayer, *Antimicrob. Agents Chemother.*, 35:2481-2485

(1991). See also Longman et al., *Rev. Infect. Dis.*, 12(Suppl. 3):S294-298 (1990).

Sterile thrombotic endocarditis is produced in New Zealand white rabbits by transaortic valvular placement of a sterile polyethylene catheter (internal diameter, 0.86 mm), which remained in place for the duration of the study. Infective
5 endocarditis is then established 48 hours after catheterization by intravenous injection of about 2×10^7 *C. albicans* blastospores. Alternatively, *C. parapsilosis* may be used. Anti-fungal therapy (chitinase or chitinase in combination with another conventional anti-fungal agent) is initiated either 24 hours before or 24 to 60 hours after fungal challenge. Therapy is continued daily for 9 or 12 days. Exemplary
10 doses of conventional anti-fungal agents are 1 mg/kg/day intravenous amphotericin B, 50 mg/kg/day or 100 mg/kg/day intravenous or intraperitoneal fluconazole. Control rabbits are given no anti-fungal agent. At sacrifice, hearts are removed and the position of the indwelling catheter verified. Cardiac vegetations from each animal are removed, pooled, weighed and homogenized in 1 ml of sterile saline. The
15 homogenate is serially diluted and quantitatively cultured on yeast potassium dextrose agar at 35°C for 48 hours. Culture-negative vegetations are considered to contain less than $2 \log_{10}$ CFU/gram on the basis of average vegetation weight.

Numerous modifications and variations of the above-described invention are expected to occur to those of skill in the art. Accordingly, only such limitations
20 as appear in the appended claims should be placed thereon.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Gray, Patrick W.
- (ii) TITLE OF INVENTION: Chitinase Materials and Methods
- (iii) NUMBER OF SEQUENCES: 15
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: Marshall, O'Toole, Gerstein, Murray & Borun
 - (B) STREET: 6300 Sears Tower, 233 South Wacker Drive
 - (C) CITY: Chicago
 - (D) STATE: Illinois
 - (E) COUNTRY: United States of America
 - (F) ZIP: 60606-6402
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER:
 - (B) FILING DATE:
 - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: Rin-Laures, Li-Hsien
 - (B) REGISTRATION NUMBER: 33,547
 - (C) REFERENCE/DOCKET NUMBER: 27866/32960
- (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: 312/474-6300
 - (B) TELEFAX: 312/474-0448
 - (C) TELEX: 25-3856

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 1636 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA
- (ix) FEATURE:
 - (A) NAME/KEY: CDS
 - (B) LOCATION: 2..1399
- (ix) FEATURE:
 - (A) NAME/KEY: mat_peptide
 - (B) LOCATION: 65..1399
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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ATC	CCA	TGG	GGC	TCT	GCT	GCA	AAA	CTG	GTC	TGC	TAC	TTC	ACC	AAC	TGG	94

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Ile	Pro	Trp	Gly	Ser	Ala	Ala	Lys	Leu	Val	Cys	Tyr	Phe	Thr	Asn	Trp		
-5						1				5					10		
GCC	CAG	TAC	AGA	CAG	GGG	GAG	GCT	CGC	TTC	CTG	CCC	AAG	GAC	TTG	GAC	142	
Ala	Gln	Tyr	Arg	Gln	Gly	Glu	Ala	Arg	Phe	Leu	Pro	Lys	Asp	Leu	Asp	25	
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CCC	AGC	CTT	TGC	ACC	CAC	CTC	ATC	TAC	GCC	TTC	GCT	GGC	ATG	ACC	AAC	190	
Pro	Ser	Leu	Cys	Thr	His	Leu	Ile	Tyr	Ala	Phe	Ala	Gly	Met	Thr	Asn	40	
			30					35					40				
CAC	CAG	CTG	AGC	ACC	ACT	GAG	TGG	AAT	GAC	GAG	ACT	CTC	TAC	CAG	GAG	238	
His	Gln	Leu	Ser	Thr	Thr	Glu	Trp	Asn	Asp	Glu	Thr	Leu	Tyr	Gln	Glu	55	
		45					50										
TTC	AAT	GGC	CTG	AAG	AAG	ATG	AAT	CCC	AAG	CTG	AAG	ACC	CTG	TTA	GCC	286	
Phe	Asn	Gly	Leu	Lys	Lys	Met	Asn	Pro	Lys	Leu	Lys	Thr	Leu	Leu	Ala	70	
	60					65											
ATC	GGA	GGC	TGG	AAT	TTC	GGC	ACT	CAG	AAG	TTC	ACA	GAT	ATG	GTA	GCC	334	
Ile	Gly	Gly	Trp	Asn	Phe	Gly	Thr	Gln	Lys	Phe	Thr	Asp	Met	Val	Ala	85	
	75				80										90		
ACG	GCC	AAC	AAC	CGT	CAG	ACC	TTT	GTC	AAC	TCG	GCC	ATC	AGG	TTT	CTG	382	
Thr	Ala	Asn	Asn	Arg	Gln	Thr	Phe	Val	Asn	Ser	Ala	Ile	Arg	Phe	Leu	100	
				95					100					105			
CGC	AAA	TAC	AGC	TTT	GAC	GGC	CTT	GAC	CTT	GAC	TGG	GAG	TAC	CCA	GGA	430	
Arg	Lys	Tyr	Ser	Phe	Asp	Gly	Leu	Asp	Leu	Asp	Trp	Glu	Tyr	Pro	Gly	110	
								115					120				
AGC	CAG	GGG	AGC	CCT	GCC	GTA	GAC	AAG	GAG	CGC	TTC	ACA	ACC	CTG	GTA	478	
Ser	Gln	Gly	Ser	Pro	Ala	Val	Asp	Lys	Glu	Arg	Phe	Thr	Thr	Leu	Val	125	
		125					130					135					
CAG	GAC	TTG	GCC	AAT	GCC	TTC	CAG	CAG	GAA	GCC	CAG	ACC	TCA	GGG	AAG	526	
Gln	Asp	Leu	Ala	Asn	Ala	Phe	Gln	Gln	Glu	Ala	Gln	Thr	Ser	Gly	Lys	140	
		140				145					150						
GAA	CGC	CTT	CTT	CTG	AGT	GCA	GCG	GTT	CCA	GCT	GGG	CAG	ACC	TAT	GTG	574	
Glu	Arg	Leu	Leu	Leu	Ser	Ala	Ala	Val	Pro	Ala	Gly	Gln	Thr	Tyr	Val	155	
					160				165						170		
GAT	GCT	GGA	TAC	GAG	GTG	GAC	AAA	ATC	GCC	CAG	AAC	CTG	GAT	TTT	GTC	622	
Asp	Ala	Gly	Tyr	Glu	Val	Asp	Lys	Ile	Ala	Gln	Asn	Leu	Asp	Phe	Val	175	
				175					180					185			
AAC	CTT	ATG	GCC	TAC	GAC	TTC	CAT	GGC	TCT	TGG	GAG	AAG	GTC	ACG	GGA	670	
Asn	Leu	Met	Ala	Tyr	Asp	Phe	His	Gly	Ser	Trp	Glu	Lys	Val	Thr	Gly	190	
			190					195					200				
CAT	AAC	AGC	CCC	CTC	TAC	AAG	AGG	CAA	GAA	GAG	AGT	GGT	GCA	GCA	GCC	718	
His	Asn	Ser	Pro	Leu	Tyr	Lys	Arg	Gln	Glu	Glu	Ser	Gly	Ala	Ala	Ala	205	
			205				210					215					
AGC	CTC	AAC	GTG	GAT	GCT	GCT	GTG	CAA	CAG	TGG	CTG	CAG	AAG	GGG	ACC	766	
Ser	Leu	Asn	Val	Asp	Ala	Ala	Val	Gln	Gln	Trp	Leu	Gln	Lys	Gly	Thr	220	
					225						230						
CCT	GCC	AGC	AAG	CTG	ATC	CTT	GGC	ATG	CCT	ACC	TAC	GGA	CGC	TCC	TTC	814	
Pro	Ala	Ser	Lys	Leu	Ile	Leu	Gly	Met	Pro	Thr	Tyr	Gly	Arg	Ser	Phe	235	
					240					245					250		
ACA	CTG	GCC	TCC	TCA	TCA	GAC	ACC	AGA	GTG	GGG	GCC	CCA	GCC	ACA	GGG	862	
Thr	Leu	Ala	Ser	Ser	Ser	Asp	Thr	Arg	Val	Gly	Ala	Pro	Ala	Thr	Gly		

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																255	260	265		
TCT	GGC	ACT	CCA	GGC	CCC	TTC	ACC	AAG	GAA	GGA	GGG	ATG	CTG	GCC	TAC	910				
Ser	Gly	Thr	Pro	Gly	Pro	Phe	Thr	Lys	Glu	Gly	Gly	Met	Leu	Ala	Tyr					
																270	275			
TAT	GAA	GTC	TGC	TCC	TGG	AAG	GGG	GCC	ACC	AAA	CAG	AGA	ATC	CAG	GAT	958				
Tyr	Glu	Val	Cys	Ser	Trp	Lys	Gly	Ala	Thr	Lys	Gln	Arg	Ile	Gln	Asp					
																285	290			
CAG	AAG	GTG	CCC	TAC	ATC	TTC	CGG	GAC	AAC	CAG	TGG	GTG	GGC	TTT	GAT	1006				
Gln	Lys	Val	Pro	Tyr	Ile	Phe	Arg	Asp	Asn	Gln	Trp	Val	Gly	Phe	Asp					
																300	305			
GAT	GTG	GAG	AGC	TTC	AAA	ACC	AAG	GTC	AGC	TAT	CTG	AAG	CAG	AAG	GGA	1054				
Asp	Val	Glu	Ser	Phe	Lys	Thr	Lys	Val	Ser	Tyr	Leu	Lys	Gln	Lys	Gly					
																315	320			
CTG	GGC	GGG	GCC	ATG	GTC	TGG	GCA	CTG	GAC	TTA	GAT	GAC	TTT	GCC	GGC	1102				
Leu	Gly	Gly	Ala	Met	Val	Trp	Ala	Leu	Asp	Leu	Asp	Asp	Phe	Ala	Gly					
																335	340			
TTC	TCC	TGC	AAC	CAG	GGC	CGA	TAC	CCC	CTC	ATC	CAG	ACG	CTA	CGG	CAG	1150				
Phe	Ser	Cys	Asn	Gln	Gly	Arg	Tyr	Pro	Leu	Ile	Gln	Thr	Leu	Arg	Gln					
																350	355			
GAA	CTG	AGT	CTT	CCA	TAC	TTG	CCT	TCA	GGC	ACC	CCA	GAG	CTT	GAA	GTT	1198				
Glu	Leu	Ser	Leu	Pro	Tyr	Leu	Pro	Ser	Gly	Thr	Pro	Glu	Leu	Glu	Val					
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CCA	AAA	CCA	GGT	CAG	CCC	TCT	GAA	CCT	GAG	CAT	GGC	CCC	AGC	CCT	GGA	1246				
Pro	Lys	Pro	Gly	Gln	Pro	Ser	Glu	Pro	Glu	His	Gly	Pro	Ser	Pro	Gly					
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CAA	GAC	ACG	TTC	TGC	CAG	GGC	AAA	GCT	GAT	GGG	CTC	TAT	CCC	AAT	CCT	1294				
Gln	Asp	Thr	Phe	Cys	Gln	Gly	Lys	Ala	Asp	Gly	Leu	Tyr	Pro	Asn	Pro					
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CGG	GAA	CGG	TCC	AGC	TTC	TAC	AGC	TGT	GCA	GCG	GGG	CGG	CTG	TTC	CAG	1342				
Arg	Glu	Arg	Ser	Ser	Phe	Tyr	Ser	Cys	Ala	Ala	Gly	Arg	Leu	Phe	Gln					
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CAA	AGC	TGC	CCG	ACA	GGC	CTG	GTG	TTC	AGC	AAC	TCC	TGC	AAA	TGC	TGC	1390				
Gln	Ser	Cys	Pro	Thr	Gly	Leu	Val	Phe	Ser	Asn	Ser	Cys	Lys	Cys	Cys					
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ACC	TGG	AAT	TGAGTCGCTA AAGCCCCTCC AGTCCCAGCT TTGAGGCTGG													1439				
Thr	Trp	Asn																		
																445				
GCCCAGGATC ACTCTACAGC CTGCCTCCTG GGTTTTCCCT GGGGGCCGCA ATCTGGCTCC																1499				
TGCAGGCCTT TCTGTGGTCT TCCTTTATCC AGGCTTTCTG CTCTCAGCCT TGCCTTCCTT																1559				
TTTTCTGGGT CTCCTGGGCT GCCCCTTTCA CTTGCAAAAT AAATCTTTGG TTTGTGCCCC																1619				
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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 466 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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Gln	Leu	Ser	Thr	Thr	Glu	Trp	Asn	Asp	Glu	Thr	Leu	Tyr	Gln	Glu	Phe
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Asn	Gly	Leu	Lys	Lys	Met	Asn	Pro	Lys	Leu	Lys	Thr	Leu	Leu	Ala	Ile
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Gly	Gly	Trp	Asn	Phe	Gly	Thr	Gln	Lys	Phe	Thr	Asp	Met	Val	Ala	Thr
				80					85					90	
Ala	Asn	Asn	Arg	Gln	Thr	Phe	Val	Asn	Ser	Ala	Ile	Arg	Phe	Leu	Arg
			95					100					105		
Lys	Tyr	Ser	Phe	Asp	Gly	Leu	Asp	Leu	Asp	Trp	Glu	Tyr	Pro	Gly	Ser
		110					115					120			
Gln	Gly	Ser	Pro	Ala	Val	Asp	Lys	Glu	Arg	Phe	Thr	Thr	Leu	Val	Gln
	125					130					135				
Asp	Leu	Ala	Asn	Ala	Phe	Gln	Gln	Glu	Ala	Gln	Thr	Ser	Gly	Lys	Glu
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Arg	Leu	Leu	Leu	Ser	Ala	Ala	Val	Pro	Ala	Gly	Gln	Thr	Tyr	Val	Asp
				160					165					170	
Ala	Gly	Tyr	Glu	Val	Asp	Lys	Ile	Ala	Gln	Asn	Leu	Asp	Phe	Val	Asn
			175					180					185		
Leu	Met	Ala	Tyr	Asp	Phe	His	Gly	Ser	Trp	Glu	Lys	Val	Thr	Gly	His
	190						195					200			
Asn	Ser	Pro	Leu	Tyr	Lys	Arg	Gln	Glu	Glu	Ser	Gly	Ala	Ala	Ala	Ser
	205					210					215				
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Ala	Ser	Lys	Leu	Ile	Leu	Gly	Met	Pro	Thr	Tyr	Gly	Arg	Ser	Phe	Thr
				240					245					250	
Leu	Ala	Ser	Ser	Ser	Asp	Thr	Arg	Val	Gly	Ala	Pro	Ala	Thr	Gly	Ser
			255					260					265		
Gly	Thr	Pro	Gly	Pro	Phe	Thr	Lys	Glu	Gly	Gly	Met	Leu	Ala	Tyr	Tyr
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Glu	Val	Cys	Ser	Trp	Lys	Gly	Ala	Thr	Lys	Gln	Arg	Ile	Gln	Asp	Gln
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Lys	Val	Pro	Tyr	Ile	Phe	Arg	Asp	Asn	Gln	Trp	Val	Gly	Phe	Asp	Asp

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300		305		310		315									
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Gly	Gly	Ala	Met	Val	Trp	Ala	Leu	Asp	Leu	Asp	Asp	Phe	Ala	Gly	Phe
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Ser	Cys	Asn	Gln	Gly	Arg	Tyr	Pro	Leu	Ile	Gln	Thr	Leu	Arg	Gln	Glu
		350					355					360			
Leu	Ser	Leu	Pro	Tyr	Leu	Pro	Ser	Gly	Thr	Pro	Glu	Leu	Glu	Val	Pro
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Lys	Pro	Gly	Gln	Pro	Ser	Glu	Pro	Glu	His	Gly	Pro	Ser	Pro	Gly	Gln
380					385					390					395
Asp	Thr	Phe	Cys	Gln	Gly	Lys	Ala	Asp	Gly	Leu	Tyr	Pro	Asn	Pro	Arg
				400					405					410	
Glu	Arg	Ser	Ser	Phe	Tyr	Ser	Cys	Ala	Ala	Gly	Arg	Leu	Phe	Gln	Gln
			415					420					425		
Ser	Cys	Pro	Thr	Gly	Leu	Val	Phe	Ser	Asn	Ser	Cys	Lys	Cys	Cys	Thr
		430					435					440			
Trp	Asn														
	445														

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 1656 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 27..1424

(ix) FEATURE:

- (A) NAME/KEY: mat_peptide
- (B) LOCATION: 90..1424

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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Phe	Met	Val	Leu	Leu	Met	Ile	Pro	Trp	Gly	Ser	Ala	Ala	Lys	Leu	Val	
			-10				-5					1				
TGC	TAC	TTC	ACC	AAC	TGG	GCC	CAG	TAC	AGA	CAG	GGG	GAG	GCT	CGC	TTC	149
Cys	Tyr	Phe	Thr	Asn	Trp	Ala	Gln	Tyr	Arg	Gln	Gly	Glu	Ala	Arg	Phe	
	5				10					15					20	
CTG	CCC	AAG	GAC	TTG	GAC	CCC	AGC	CTT	TGC	ACC	CAC	CTC	ATC	TAC	GCC	197
Leu	Pro	Lys	Asp	Leu	Asp	Pro	Ser	Leu	Cys	Thr	His	Leu	Ile	Tyr	Ala	
			25						30						35	

TTC	GCT	GGC	ATG	ACC	AAC	CAC	CAG	CTG	AGC	ACC	ACT	GAG	TGG	AAT	GAC	245
Phe	Ala	Gly	Met	Thr	Asn	His	Gln	Leu	Ser	Thr	Thr	Glu	Trp	Asn	Asp	
			40					45					50			
GAG	ACT	CTC	TAC	CAG	GAG	TTC	AAT	GGC	CTG	AAG	AAG	ATG	AAT	CCC	AAG	293
Glu	Thr	Leu	Tyr	Gln	Glu	Phe	Asn	Gly	Leu	Lys	Lys	Met	Asn	Pro	Lys	
		55					60					65				
CTG	AAG	ACC	CTG	TTA	GCC	ATC	GGA	GGC	TGG	AAT	TTC	AGC	ACT	CAG	AAG	341
Leu	Lys	Thr	Leu	Leu	Ala	Ile	Gly	Gly	Trp	Asn	Phe	Ser	Thr	Gln	Lys	
	70					75					80					
TTC	ACA	GAT	ATG	GTA	GCC	ACG	GCC	AAC	AAC	CGT	CAG	ACC	TTT	GTC	AAC	389
Phe	Thr	Asp	Met	Val	Ala	Thr	Ala	Asn	Asn	Arg	Gln	Thr	Phe	Val	Asn	
	85				90					95					100	
TCG	GCC	ATC	AGG	TTT	CTG	CGC	AAA	TAC	AGC	TTT	GAC	GGC	CTT	GAC	CTT	437
Ser	Ala	Ile	Arg	Phe	Leu	Arg	Lys	Tyr	Ser	Phe	Asp	Gly	Leu	Asp	Leu	
				105					110					115		
GAC	TGG	GAG	TAC	CCA	GGA	AGC	CAG	GGG	AGC	CCT	GCC	GTA	GAC	AAG	GAG	485
Asp	Trp	Glu	Tyr	Pro	Gly	Ser	Gln	Gly	Ser	Pro	Ala	Val	Asp	Lys	Glu	
			120					125					130			
CGC	TTC	ACA	ACC	CTG	GTA	CAG	GAC	TTG	GCC	AAT	GCC	TTC	CAG	CAG	GAA	533
Arg	Phe	Thr	Thr	Leu	Val	Gln	Asp	Leu	Ala	Asn	Ala	Phe	Gln	Gln	Glu	
		135					140					145				
GCC	CAG	ACC	TCA	GGG	AAG	GAA	CGC	CTT	CTT	CTG	AGT	GCA	GCG	GTT	CCA	581
Ala	Gln	Thr	Ser	Gly	Lys	Glu	Arg	Leu	Leu	Leu	Ser	Ala	Ala	Val	Pro	
	150					155					160					
GCT	GGG	CAG	ACC	TAT	GTG	GAT	GCT	GGA	TAC	GAG	GTG	GAC	AAA	ATC	GCC	629
Ala	Gly	Gln	Thr	Tyr	Val	Asp	Ala	Gly	Tyr	Glu	Val	Asp	Lys	Ile	Ala	
	165				170					175					180	
CAG	AAC	CTG	GAT	TTT	GTC	AAC	CTT	ATG	GCC	TAC	GAC	TTC	CAT	GGC	TCT	677
Gln	Asn	Leu	Asp	Phe	Val	Asn	Leu	Met	Ala	Tyr	Asp	Phe	His	Gly	Ser	
				185					190					195		
TGG	GAG	AAG	GTC	ACG	GGA	CAT	AAC	AGC	CCC	CTC	TAC	AAG	AGG	CAA	GAA	725
Trp	Glu	Lys	Val	Thr	Gly	His	Asn	Ser	Pro	Leu	Tyr	Lys	Arg	Gln	Glu	
			200					205					210			
GAG	AGT	GGT	GCA	GCA	GCC	AGC	CTC	AAC	GTG	GAT	GCT	GCT	GTG	CAA	CAG	773
Glu	Ser	Gly	Ala	Ala	Ala	Ser	Leu	Asn	Val	Asp	Ala	Ala	Val	Gln	Gln	
		215					220					225				
TGG	CTG	CAG	AAG	GGG	ACC	CCT	GCC	AGC	AAG	CTG	ATC	CTT	GGC	ATG	CCT	821
Trp	Leu	Gln	Lys	Gly	Thr	Pro	Ala	Ser	Lys	Leu	Ile	Leu	Gly	Met	Pro	
	230					235					240					
ACC	TAC	GGA	CGC	TCC	TTC	ACA	CTG	GCC	TCC	TCA	TCA	GAC	ACC	AGA	GTG	869
Thr	Tyr	Gly	Arg	Ser	Phe	Thr	Leu	Ala	Ser	Ser	Ser	Asp	Thr	Arg	Val	
	245				250					255					260	
GGG	GCC	CCA	GCC	ACA	GGG	TCT	GGC	ACT	CCA	GGC	CCC	TTC	ACC	AAG	GAA	917
Gly	Ala	Pro	Ala	Thr	Gly	Ser	Gly	Thr	Pro	Gly	Pro	Phe	Thr	Lys	Glu	
			265						270					275		
GGA	GGG	ATG	CTG	GCC	TAC	TAT	GAA	GTC	TGC	TCC	TGG	AAG	GGG	GCC	ACC	965
Gly	Gly	Met	Leu	Ala	Tyr	Tyr	Glu	Val	Cys	Ser	Trp	Lys	Gly	Ala	Thr	
			280					285					290			
AAA	CAG	AGA	ATC	CAG	GAT	CAG	AAG	GTG	CCC	TAC	ATC	TTC	CGG	GAC	AAC	1013

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Lys	Gln	Arg	Ile	Gln	Asp	Gln	Lys	Val	Pro	Tyr	Ile	Phe	Arg	Asp	Asn	
		295					300					305				
CAG	TGG	GTG	GGC	TTT	GAT	GAT	GTG	GAG	AGC	TTC	AAA	ACC	AAG	GTC	AGC	1061
Gln	Trp	Val	Gly	Phe	Asp	Asp	Val	Glu	Ser	Phe	Lys	Thr	Lys	Val	Ser	
	310					315					320					
TAT	CTG	AAG	CAG	AAG	GGA	CTG	GGC	GGG	GCC	ATG	GTC	TGG	GCA	CTG	GAC	1109
Tyr	Leu	Lys	Gln	Lys	Gly	Leu	Gly	Gly	Ala	Met	Val	Trp	Ala	Leu	Asp	
	325				330					335					340	
TTA	GAT	GAC	TTT	GCC	GGC	TTC	TCC	TGC	AAC	CAG	GGC	CGA	TAC	CCC	CTC	1157
Leu	Asp	Asp	Phe	Ala	Gly	Phe	Ser	Cys	Asn	Gln	Gly	Arg	Tyr	Pro	Leu	
				345					350					355		
ATC	CAG	ACG	CTA	CGG	CAG	GAA	CTG	AGT	CTT	CCA	TAC	TTG	CCT	TCA	GGC	1205
Ile	Gln	Thr	Leu	Arg	Gln	Glu	Leu	Ser	Leu	Pro	Tyr	Leu	Pro	Ser	Gly	
			360					365					370			
ACC	CCA	GAG	CTT	GAA	GTT	CCA	AAA	CCA	GGT	CAG	CCC	TCT	GAA	CCT	GAG	1253
Thr	Pro	Glu	Leu	Glu	Val	Pro	Lys	Pro	Gly	Gln	Pro	Ser	Glu	Pro	Glu	
		375					380					385				
CAT	GGC	CCC	AGC	CCT	GGA	CAA	GAC	ACG	TTC	TGC	CAG	GGC	AAA	GCT	GAT	1301
His	Gly	Pro	Ser	Pro	Gly	Gln	Asp	Thr	Phe	Cys	Gln	Gly	Lys	Ala	Asp	
	390					395					400					
GGG	CTC	TAT	CCC	AAT	CCT	CGG	GAA	CGG	TCC	AGC	TTC	TAC	AGC	TGT	GCA	1349
Gly	Leu	Tyr	Pro	Asn	Pro	Arg	Glu	Arg	Ser	Ser	Phe	Tyr	Ser	Cys	Ala	
	405				410				415						420	
GCG	GGG	CGG	CTG	TTC	CAG	CAA	AGC	TGC	CCG	ACA	GGC	CTG	GTG	TTC	AGC	1397
Ala	Gly	Arg	Leu	Phe	Gln	Gln	Ser	Cys	Pro	Thr	Gly	Leu	Val	Phe	Ser	
			425						430					435		
AAC	TCC	TGC	AAA	TGC	TGC	ACC	TGG	AAT	TGAGTCGCTA	AAGCCCCCTCC						1444
Asn	Ser	Cys	Lys	Cys	Cys	Thr	Trp	Asn								
			440				445									
AGTCCCAGCT	TTGAGGCTGG	GCCCAGGATC	ACTCTACAGC	CTGCCTCCTG	GGTTTTCCCT											1504
GGGGGCCGCA	ATCTGGCTCC	TGCAGGCCTT	TCTGTGGTCT	TCCTTTATCC	AGGCTTTCTG											1564
CTCTCAGCCT	TGCCCTCCTT	TTTTCTGGGT	CTCCTGGGCT	GCCCCCTTTCA	CTTGCAAAAT											1624
AAATCTTTGG	TTTGTGCCCC	TCAAAAAAAA	AA													1656

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 466 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met	Val	Arg	Ser	Val	Ala	Trp	Ala	Gly	Phe	Met	Val	Leu	Leu	Met	Ile	
-21	-20					-15					-10					
Pro	Trp	Gly	Ser	Ala	Ala	Lys	Leu	Val	Cys	Tyr	Phe	Thr	Asn	Trp	Ala	
-5					1				5					10		
Gln	Tyr	Arg	Gln	Gly	Glu	Ala	Arg	Phe	Leu	Pro	Lys	Asp	Leu	Asp	Pro	

15					20					25					
Ser	Leu	Cys	Thr	His	Leu	Ile	Tyr	Ala	Phe	Ala	Gly	Met	Thr	Asn	His
		30					35					40			
Gln	Leu	Ser	Thr	Thr	Glu	Trp	Asn	Asp	Glu	Thr	Leu	Tyr	Gln	Glu	Phe
	45					50					55				
Asn	Gly	Leu	Lys	Lys	Met	Asn	Pro	Lys	Leu	Lys	Thr	Leu	Leu	Ala	Ile
	60				65					70					75
Gly	Gly	Trp	Asn	Phe	Ser	Thr	Gln	Lys	Phe	Thr	Asp	Met	Val	Ala	Thr
				80					85					90	
Ala	Asn	Asn	Arg	Gln	Thr	Phe	Val	Asn	Ser	Ala	Ile	Arg	Phe	Leu	Arg
			95					100					105		
Lys	Tyr	Ser	Phe	Asp	Gly	Leu	Asp	Leu	Asp	Trp	Glu	Tyr	Pro	Gly	Ser
		110					115					120			
Gln	Gly	Ser	Pro	Ala	Val	Asp	Lys	Glu	Arg	Phe	Thr	Thr	Leu	Val	Gln
	125					130					135				
Asp	Leu	Ala	Asn	Ala	Phe	Gln	Gln	Glu	Ala	Gln	Thr	Ser	Gly	Lys	Glu
	140				145					150					155
Arg	Leu	Leu	Leu	Ser	Ala	Ala	Val	Pro	Ala	Gly	Gln	Thr	Tyr	Val	Asp
				160					165					170	
Ala	Gly	Tyr	Glu	Val	Asp	Lys	Ile	Ala	Gln	Asn	Leu	Asp	Phe	Val	Asn
			175					180					185		
Leu	Met	Ala	Tyr	Asp	Phe	His	Gly	Ser	Trp	Glu	Lys	Val	Thr	Gly	His
		190					195					200			
Asn	Ser	Pro	Leu	Tyr	Lys	Arg	Gln	Glu	Glu	Ser	Gly	Ala	Ala	Ala	Ser
		205				210					215				
Leu	Asn	Val	Asp	Ala	Ala	Val	Gln	Gln	Trp	Leu	Gln	Lys	Gly	Thr	Pro
	220				225					230					235
Ala	Ser	Lys	Leu	Ile	Leu	Gly	Met	Pro	Thr	Tyr	Gly	Arg	Ser	Phe	Thr
				240					245					250	
Leu	Ala	Ser	Ser	Ser	Asp	Thr	Arg	Val	Gly	Ala	Pro	Ala	Thr	Gly	Ser
			255					260					265		
Gly	Thr	Pro	Gly	Pro	Phe	Thr	Lys	Glu	Gly	Gly	Met	Leu	Ala	Tyr	Tyr
		270					275					280			
Glu	Val	Cys	Ser	Trp	Lys	Gly	Ala	Thr	Lys	Gln	Arg	Ile	Gln	Asp	Gln
	285					290					295				
Lys	Val	Pro	Tyr	Ile	Phe	Arg	Asp	Asn	Gln	Trp	Val	Gly	Phe	Asp	Asp
	300				305					310				315	
Val	Glu	Ser	Phe	Lys	Thr	Lys	Val	Ser	Tyr	Leu	Lys	Gln	Lys	Gly	Leu
				320					325					330	
Gly	Gly	Ala	Met	Val	Trp	Ala	Leu	Asp	Leu	Asp	Asp	Phe	Ala	Gly	Phe
			335					340					345		
Ser	Cys	Asn	Gln	Gly	Arg	Tyr	Pro	Leu	Ile	Gln	Thr	Leu	Arg	Gln	Glu
		350					355					360			

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Leu Ser Leu Pro Tyr Leu Pro Ser Gly Thr Pro Glu Leu Glu Val Pro
 365 370 375
 Lys Pro Gly Gln Pro Ser Glu Pro Glu His Gly Pro Ser Pro Gly Gln
 380 385 390 395
 Asp Thr Phe Cys Gln Gly Lys Ala Asp Gly Leu Tyr Pro Asn Pro Arg
 400 405 410
 Glu Arg Ser Ser Phe Tyr Ser Cys Ala Ala Gly Arg Leu Phe Gln Gln
 415 420 425
 Ser Cys Pro Thr Gly Leu Val Phe Ser Asn Ser Cys Lys Cys Cys Thr
 430 435 440
 Trp Asn
 445

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 18 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GACACTATAG AATAGGGC

18

(2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 51 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

TGGGATCATC AGCAGGACCA TGAAACCTGC CCAGGCCACA GACCGCACCA T

51

(2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 40 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

TACATCTAGA ATTATGGCAA AACTGGTCTG CTACTTCACC

40

(2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 34 base pairs
 - (B) TYPE: nucleic acid

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- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

AGATCTAACC TTAGGTGCCT GAAGACAAGT ATGG

34

(2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 29 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

TACAGAATTC TTATTCACAT CCGGCCCTG

29

(2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 34 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

TACATCTAGA CTCCATCCAG AAAACAGGT ATGG

34

(2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 30 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

TCTAGAGTCG ACCTGCAGGC ATGCAAGCTT

30

(2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 50 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CGCAAGCTTG AGAGCTCCGT TCCGCCACAT GGTGCGGTCT GTGGCCTGGG

50

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(2) INFORMATION FOR SEQ ID NO:13:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 32 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GACTCTAGAC TAGGTGCCTG AAGGCAAGTA TG

32

(2) INFORMATION FOR SEQ ID NO:14:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 373 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Ala	Lys	Leu	Val	Cys	Tyr	Phe	Thr	Asn	Trp	Ala	Gln	Tyr	Arg	Gln	Gly	1	5	10	15
Glu	Ala	Arg	Phe	Leu	Pro	Lys	Asp	Leu	Asp	Pro	Ser	Leu	Cys	Thr	His	20	25	30	
Leu	Ile	Tyr	Ala	Phe	Ala	Gly	Met	Thr	Asn	His	Gln	Leu	Ser	Thr	Thr	35	40	45	
Glu	Trp	Asn	Asp	Glu	Thr	Leu	Tyr	Gln	Glu	Phe	Asn	Gly	Leu	Lys	Lys	50	55	60	
Met	Asn	Pro	Lys	Leu	Lys	Thr	Leu	Leu	Ala	Ile	Gly	Gly	Trp	Asn	Phe	65	70	75	80
Gly	Thr	Gln	Lys	Phe	Thr	Asp	Met	Val	Ala	Thr	Ala	Asn	Asn	Arg	Gln	85	90	95	
Thr	Phe	Val	Asn	Ser	Ala	Ile	Arg	Phe	Leu	Arg	Lys	Tyr	Ser	Phe	Asp	100	105	110	
Gly	Leu	Asp	Leu	Asp	Trp	Glu	Tyr	Pro	Gly	Ser	Gln	Gly	Ser	Pro	Ala	115	120	125	
Val	Asp	Lys	Glu	Arg	Phe	Thr	Thr	Leu	Val	Gln	Asp	Leu	Ala	Asn	Ala	130	135	140	
Phe	Gln	Gln	Glu	Ala	Gln	Thr	Ser	Gly	Lys	Glu	Arg	Leu	Leu	Leu	Ser	145	150	155	160
Ala	Ala	Val	Pro	Ala	Gly	Gln	Thr	Tyr	Val	Asp	Ala	Gly	Tyr	Glu	Val	165	170	175	
Asp	Lys	Ile	Ala	Gln	Asn	Leu	Asp	Phe	Val	Asn	Leu	Met	Ala	Tyr	Asp	180	185	190	
Phe	His	Gly	Ser	Trp	Glu	Lys	Val	Thr	Gly	His	Asn	Ser	Pro	Leu	Tyr	195	200	205	

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Lys Arg Gln Glu Glu Ser Gly Ala Ala Ala Ser Leu Asn Val Asp Ala
 210 215 220
 Ala Val Gln Gln Trp Leu Gln Lys Gly Thr Pro Ala Ser Lys Leu Ile
 225 230 235 240
 Leu Gly Met Pro Thr Tyr Gly Arg Ser Phe Thr Leu Ala Ser Ser Ser
 245 250 255
 Asp Thr Arg Val Gly Ala Pro Ala Thr Gly Ser Gly Thr Pro Gly Pro
 260 265 270
 Phe Thr Lys Glu Gly Gly Met Leu Ala Tyr Tyr Glu Val Cys Ser Trp
 275 280 285
 Lys Gly Ala Thr Lys Gln Arg Ile Gln Asp Gln Lys Val Pro Tyr Ile
 290 295 300
 Phe Arg Asp Asn Gln Trp Val Gly Phe Asp Asp Val Glu Ser Phe Lys
 305 310 315 320
 Thr Lys Val Ser Tyr Leu Lys Gln Lys Gly Leu Gly Gly Ala Met Val
 325 330 335
 Trp Ala Leu Asp Leu Asp Asp Phe Ala Gly Phe Ser Cys Asn Gln Gly
 340 345 350
 Arg Tyr Pro Leu Ile Gln Thr Leu Arg Gln Glu Leu Ser Leu Pro Tyr
 355 360 365
 Leu Pro Ser Gly Thr
 370

(2) INFORMATION FOR SEQ ID NO:15:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 373 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Ala Lys Leu Val Cys Tyr Phe Thr Asn Trp Ala Gln Tyr Arg Gln Gly
 1 5 10 15
 Glu Ala Arg Phe Leu Pro Lys Asp Leu Asp Pro Ser Leu Cys Thr His
 20 25 30
 Leu Ile Tyr Ala Phe Ala Gly Met Thr Asn His Gln Leu Ser Thr Thr
 35 40 45
 Glu Trp Asn Asp Glu Thr Leu Tyr Gln Glu Phe Asn Gly Leu Lys Lys
 50 55 60
 Met Asn Pro Lys Leu Lys Thr Leu Leu Ala Ile Gly Gly Trp Asn Phe
 65 70 75 80
 Gly Thr Gln Lys Phe Thr Asp Met Val Ala Thr Ala Asn Asn Arg Gln
 85 90 95
 Thr Phe Val Asn Ser Ala Ile Arg Phe Leu Arg Lys Tyr Ser Phe Asp
 100 105 110

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CLAIMS

What is claimed is:

1. A purified, isolated polynucleotide encoding the human chitinase amino acid sequence of SEQ ID NO: 2.
2. The polynucleotide of claim 1 which is a DNA.
3. The DNA of claim 2 comprising the protein coding nucleotides of SEQ ID NO: 1.
4. A purified, isolated polynucleotide encoding amino acids 1 to 445 of SEQ ID NO: 2.
5. The polynucleotide of claim 4 which is a DNA.
6. The DNA of claim 5 comprising nucleotides 65 to 1402 of SEQ ID NO: 1.
7. A purified, isolated polynucleotide encoding the human chitinase amino acid sequence of SEQ ID NO: 4.
8. The polynucleotide of claim 7 which is a DNA.
9. The DNA of claim 8 comprising the protein coding nucleotides of SEQ ID NO: 3.
10. A purified, isolated polynucleotide encoding amino acids 1 to 445 of SEQ ID NO: 4.
11. The polynucleotide of claim 10 which is a DNA.

12. The DNA of claim 11 comprising nucleotides 90 to 1427 of SEQ ID NO: 3.

13. A purified, isolated polynucleotide encoding human chitinase selected from the group consisting of:

(a) a double-stranded DNA comprising the protein coding portions of the sequence set out in SEQ ID NO: 1;

(b) a DNA which hybridizes under stringent conditions to a non-coding strand of the DNA of (a); and

(c) a DNA which, but for the redundancy of the genetic code, would hybridize under stringent conditions to a non-coding strand of DNA sequence of (a) or (b).

14. The polynucleotide of claim 13 which is a DNA.

15. A vector comprising the DNA of claim 2, 3, 5, 6, 8, 9, 11, 12, or 14.

16. The vector of claim 15 that is an expression vector, wherein the DNA is operatively linked to an expression control DNA sequence.

17. A host cell stably transformed or transfected with the DNA of claim 2, 3, 5, 6, 8, 9, 11, 12, or 14 in a manner allowing the expression in said host cell of human chitinase.

18. A method for producing human chitinase comprising culturing the host cell of claim 17 in a nutrient medium and isolating human chitinase from said host cell or said nutrient medium.

19. A purified, isolated polypeptide produced by the method of claim 18.

20. A purified, isolated polypeptide comprising the human chitinase amino acid sequence of SEQ ID NO: 2.

21. A purified, isolated polypeptide comprising the human chitinase amino acid sequence of SEQ ID NO: 4.

22. A purified, isolated polypeptide comprising human chitinase amino acids 1 to 445 of SEQ ID NO: 2.

23. A purified, isolated polypeptide comprising human chitinase amino acids 1 to 445 of SEQ ID NO: 4.

24. A human chitinase fragment lacking from 1 to about 72 C-terminal amino acid residues of mature human chitinase.

25. The human chitinase fragment of SEQ ID NO: 14.

26. A purified, isolated polynucleotide comprising a polynucleotide sequence encoding the amino acid sequence of SEQ ID NO: 14.

27. The polynucleotide of claim 26 which is a DNA.

28. The human chitinase analog of SEQ ID NO: 15.

29. A purified, isolated polynucleotide encoding the amino acid sequence of SEQ ID NO: 15.

30. A hybridoma cell line producing a monoclonal antibody that is specifically reactive with the polypeptide of claims 19, 20, 21, 22, 23 or 28.

31. The monoclonal antibody produced by the hybridoma of claim 30.

ABSTRACT

The present invention provides purified and isolated polynucleotide sequences encoding human chitinase. Also provided are materials and methods for the recombinant production of human chitinase products which are expected to be useful as products for treating fungal infections or for development of products useful for treating the same.

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DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that my residence, post office address and citizenship are as stated below next to my name. I believe that I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled **CHITINASE MATERIALS AND METHODS**, the specification of which (check one): ☐ is attached hereto; ☒ was filed on June 14, 1996 as Application Serial No. 08/663,618 and was amended on _____ (if applicable); ☐ was filed as PCT International Application No. _____ on _____ and was amended under Article 19 on _____ (if applicable). I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment(s) referred to above. I acknowledge the duty to disclose to the Patent and Trademark Office all information known to me to be material to patentability as defined in 37 C.F.R. §1.56.

I hereby claim foreign priority benefits under 35 U.S.C. §119 of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed:

Priority Claimed

(Application Serial Number)	(Country)	(Day/Month/Year Filed)	Yes	No
(Application Serial Number)	(Country)	(Day/Month/Year Filed)	Yes	No

I hereby claim the benefit under 35 U.S.C. §119(e) of any United States provisional application(s) listed below:

(Application Serial Number)	(Day/Month/Year Filed)
(Application Serial Number)	(Day/Month/Year Filed)

I hereby claim the benefit under 35 U.S.C. §120 of any United States application(s) or PCT international application(s) designating the United States of America listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior application(s) in the manner provided by the first paragraph of 35 U.S.C. §112, I acknowledge the duty to disclose to the Office all information known to me to be material to patentability as defined in 37 C.F.R. §1.56 which occurred between the filing date of the prior application(s) and the national or PCT international filing date of this application:

(Application Serial Number)	(Day/Month/Year Filed)	(Status-Patented, Pending or Abandoned)
(Application Serial Number)	(Day/Month/Year Filed)	(Status-Patented, Pending or Abandoned)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. §1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

POWER OF ATTORNEY: I hereby appoint as my attorneys, with full powers of substitution and revocation, to prosecute this application and transact all business in the Patent and Trademark Office connected therewith:

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☐ See second page for additional inventor(s)

See reverse for relevant rules & statutes

**PATENT**

Attorney's Docket No: 27866/32960

Applicant or Patentee: Patrick W. Gray
Serial or Patent No: 08/663,618
Filed or Issued: June 14, 1996
For: Chitinase Materials and Methods

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS (37 CFR 1.9(f) and 1.27(c)) -- SMALL BUSINESS CONCERN

I hereby declare that I am

The owner of the small business concern identified below:

- ☒ An official of the small business concern empowered to act on behalf of the concern identified below:

NAME OF CONCERN ICOS Corporation

ADDRESS OF BUSINESS 22021 20th Avenue, S.E.,
Bothell, Washington 98021

I hereby declare that the above-identified small business concern qualifies as a small business concern as defined in 13 CFR 121.12, and reproduced in 37 CFR 1.9(d), for purposes of paying reduced fees under Section 41(a) and (b) of Title 35, United States Code, in that the number of employees of the concern, including those of its affiliates, does not exceed 500 persons. For purposes of this statement, (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the persons employed on a full-time, part-time or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third-party or parties controls or has the power to control both.

I hereby declare that rights under contract or law have been conveyed to, and remain with, the small business concern identified above with regard to the invention, entitled Chitinase Materials and Methods, by inventor(s) Patrick W. Gray,

~~SE10005 11/14/96 082828~~
described in

The specification filed herewith.

- ☒ Application Serial No. 08/663,618, filed June 14, 1996.

Patent No. _____, issued _____.

If the rights held by the above-identified small business concern are not exclusive,

08663618-082896

***NOTE:**

Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27).

NAME: _____

ADDRESS: _____

☐ INDIVIDUAL☐ SMALL BUSINESS CONCERN☐ NONPROFIT ORGANIZATION

NAME: _____

ADDRESS: _____

☐ INDIVIDUAL☐ SMALL BUSINESS CONCERN☐ NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b)).

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING:

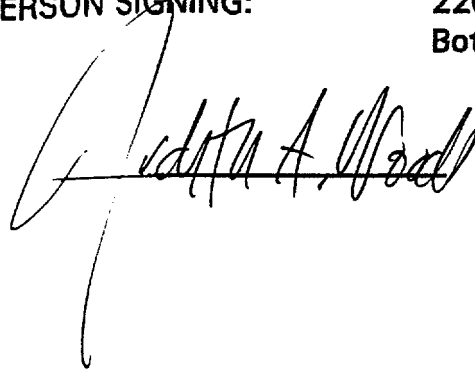
Judith Woods

NAME OF PERSON OTHER THAN OWNER: Patent and Licensing Counsel

ADDRESS OF PERSON SIGNING:

22021 20th Avenue, S.E.,
Bothell, Washington, 98021

SIGNATURE:



Date

8-13-96

08663618-082806

7. **Deposit Account and Refund Authorization**

The Commissioner is hereby authorized to charge any deficiency in the amount enclosed or any additional fees which may be required during the pendency of this application under 37 CFR 1.16 or 37 CFR 1.17 or under other applicable rules (except payment of issue fees), to Deposit Account No. 13-2855. A copy of this Transmittal is enclosed.

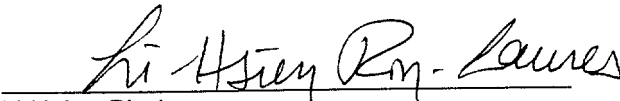
Please refund any overpayment to Marshall, O'Toole, Gerstein, Murray & Borun at the address below.

Please direct all future communications to Li-Hsien Rin-Laures, at the address below.

Respectfully submitted,

MARSHALL, O'TOOLE, GERSTEIN,
MURRAY & BORUN
6300 Sears Tower
233 South Wacker Drive
Chicago, Illinois 60606-6402
(312) 474-6300

By:


Li-Hsien Rin-Laures
Reg. No: 33,547

June 14, 1996

00663618.082896



PATENT

Attorney's Docket No: 27866/32960

Applicant or Patentee: Patrick W. Gray
Serial or Patent No: 08/663,618
Filed or Issued: June 14, 1996
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described in

The specification filed herewith.

- ☒ Application Serial No. 08/663,618, filed June 14, 1996.

Patent No. _____, issued _____.

If the rights held by the above-identified small business concern are not exclusive, each individual, concern or organization having rights to the invention is listed below* and no rights to the invention are held by any person, other than the inventor, who could not qualify as a small business concern under 37 CFR 1.9(d) or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

08663618-082896

***NOTE:** *Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27).*

NAME: _____
ADDRESS: _____
☐ INDIVIDUAL ☐ SMALL BUSINESS CONCERN ☐ NONPROFIT ORGANIZATION

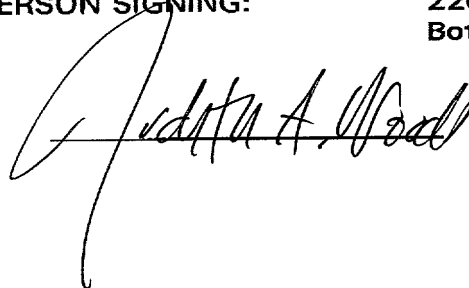
NAME: _____
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NAME OF PERSON SIGNING: Judith Woods
TITLE OF PERSON OTHER THAN OWNER: Patent and Licensing Counsel
ADDRESS OF PERSON SIGNING: 22021 20th Avenue, S.E.,
Bothell, Washington, 98021

SIGNATURE:



Date 8-13-96